



Organisation
Mondiale
de la Santé
Animale

World
Organisation
for Animal
Health

Organización
Mundial
de Sanidad
Animal

Original: English
February 2020

**REPORT OF THE MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION**

Paris, 19–26 February 2020

EU comment

The EU would like to commend the OIE Aquatic Animal Health Standards Commission for its work and for having taken into consideration EU comments on the Aquatic Code and Manual submitted previously.

The EU notes the reply from the Aquatic Animals Commission to some of the previous EU comments and the justification why some of those comments have not been taken into consideration.

The EU only has few specific comments on this report of the February 2020 meeting of the Aquatic Animals Commission (inserted in the text below) and in general supports the proposed changes to chapters and articles presented therein.

The EU would like to stress again its continued commitment to participate in the work of the OIE and to offer all technical support needed by the Aquatic Animals Commission and its ad hoc groups for future work on the Aquatic Code and Manual.

The COVID-19 pandemic has made it necessary to review the arrangements for Members' participation in international meetings, and in particular the 88th General Session of the World Assembly of Delegates of the OIE. In this context, the OIE Council held an extraordinary meeting on 6 March 2020, and decided in agreement with the Director General, that the OIE General Session for May 2020 would be restricted to Wednesday 27 May 2020 and be limited to the consideration of institutional matters (elections) and administrative matters (vote on the budget), so as to ensure the institutional functioning of the Organisation; and technical matters that require the approbation of the World Assembly on a regular annual basis, when that approbation is supported by mechanisms of technical and procedural oversight previously agreed by the World Assembly (Official Recognition of Status; Reference Centres; Register of Diagnostic Kits).

As a consequence, no new or amended chapters in the *Aquatic Animal Health Code*, the *Terrestrial Animal Health Code*, the *Manual of Diagnostic Tests for Aquatic Animals* or the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* will be proposed for adoption in 2020. Chapters that were to be proposed for adoption in 2020 will be proposed for adoption in May 2021.

To ensure a consistent approach across all three Specialist Commissions presenting international standards to the World Assembly, the OIE has decided to take the following approach:

1. All relevant texts that were to be proposed for adoption in May 2020 will be circulated in the respective Specialist Commission's February 2020 report noting that adoption has been postponed until May 2021, and that they will be open for one round of comments.
2. Only substantive comments that have not been submitted before will be considered.
3. The deadline for comments for relevant Aquatic Animals Commission Annexes is 10 July 2020.
4. Each Commission will consider such comments at its September 2020 or February 2021 meetings thereby spreading out this work and enabling them to progress other work items.
5. The texts (incorporating any revisions resulting from this process) will be included in the relevant Commission's February 2021 reports, to be proposed for adoption in May 2021.
6. This process does not alter the regular process applying to other chapters being circulated for comments.

The OIE Aquatic Animal Health Standards Commission (Aquatic Animals Commission) met at OIE Headquarters in Paris from 19 to 26 February 2020. The list of participants is presented in [Annex 1](#).

The Aquatic Animals Commission thanked the following Members for providing written comments on draft texts for the OIE *Aquatic Animal Health Code* (hereinafter referred to as the *Aquatic Code*) and OIE *Manual of Diagnostic Tests for Aquatic Animals* (hereinafter referred to as the *Aquatic Manual*) circulated in the Commission's September 2019 meeting report: Argentina, Australia, Canada, Chile, Chinese Taipei, China (People's Rep. of), Cuba, Ecuador, Japan, Korea (Rep. of), New Caledonia, New Zealand, Nicaragua, Norway, Peru, Singapore, Thailand, the United States of America (the USA), the Member States of European Union (EU) and the African Union Interafrican Bureau for Animal Resources (AU-IBAR) on behalf of African Member Countries of the OIE.

The Commission considered all comments that were submitted on time and were supported by a rationale. The Commission made amendments to draft texts, where relevant, in the usual manner by 'double underline' and '~~striketrough~~'. In the Annexes, amendments proposed at this meeting are highlighted with a coloured background in order to distinguish them from those made previously. The Commission did not consider comments where a rationale had not been provided or that were difficult to interpret.

The Commission encourages Members to consider relevant information in previous Commission and *ad hoc* Group reports when preparing comments, especially on longstanding issues. These reports are available on the [OIE Website](#).

The Table of Contents below lists the meeting agenda items and provides links to the items. Members should note that texts in **Annexes 2 to 7 and 11 to 14** that were to be proposed for adoption in May 2020, will be proposed for adoption in May 2021 and are open for an additional round of comments. As these texts have already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously. **Annexes 8 to 9 and 15 to 16** are presented for Member comments. **Annex 10** is presented for Member information.

Comments on **Annexes 2 to 16** of this report must reach OIE Headquarters by the **10 July 2020** to be considered at the September 2020 meeting of the Aquatic Animals Commission. Comments received after this due date will not be submitted to the Commission for its consideration.

All comments should be sent to the OIE Standards Department at: standards.dept@oie.int.

Comments should be submitted as Word files rather than pdf files because pdf files are difficult to incorporate into the Commission's working documents.

Comments should be presented in the relevant Annex, and include new proposed text, supported by a structured rationale or by published scientific references. Proposed deletions should be indicated in '~~striketrough~~' and proposed additions with 'double underline'. Members should not use the automatic 'track-changes' function provided by Word processing software, as such changes are lost in the process of collating Members' submissions into the Aquatic Animals Commission's working documents. Members are also requested not to reproduce the full text of a chapter as this makes it easy to miss comments while preparing the working documents.

The Aquatic Animals Commission strongly encourages Members to participate in the development of the OIE's international standards by submitting comments on this report and participate in the process of adoption at the General Session.

Table of Contents

1. WELCOME FROM THE DEPUTY DIRECTOR GENERAL	4
2. ADOPTED AGENDA	4
3. MEETING WITH THE DIRECTOR GENERAL	4
4. COOPERATION WITH OTHER SPECIALIST COMMISSIONS	5

5. OIE AQUATIC ANIMAL HEALTH STRATEGY	5
6. WORK PLAN OF THE AQUATIC ANIMALS COMMISSION	5
7. OIE AQUATIC ANIMAL HEALTH CODE	6
7.1. Texts to be proposed for adoption in May 2021	6
7.1.1. New draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X.)	6
7.1.2. Listing of infection with decapod iridescent virus 1 (DIV1) — Revised Article 1.3.3. of Chapter 1.3.....	11
7.1.3. Model Article 10.X.13. for the fish disease-specific Chapters 10.5, 10.6. and 10.10. (and Article 10.4.17. for Chapter 10.4.)	11
7.1.4. Article 10.9.2. of Chapter 10.9. Infection with spring viraemia of carp virus.....	12
7.1.5. Article 10.10.2. of Chapter 10.10. Infection with viral haemorrhagic septicaemia virus	13
7.1.6. Glossary definitions for ‘Aquatic animal waste’ and ‘aquatic animal products’	14
7.2. Texts for Member comments	15
7.2.1. Revised Glossary definition for ‘vector’	15
7.2.2. Approaches for determining periods required to demonstrate disease freedom.....	15
7.3. Texts for Member information	16
7.3.1. De-listing of infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) — Revised Article 1.3.3. of Chapter 1.3.....	16
7.3.2. New draft chapters on emergency disease preparedness and disease outbreak management.....	16
7.3.3. Infection with Carp edema virus (CEV).....	17
8. OIE MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS	17
8.1. Status of Aquatic Manual revisions	17
8.2. Texts to be proposed for adoption in May 2021	17
8.2.1. Infection with spring viraemia of carp virus (Chapter 2.3.9.)	18
8.2.2. Infection with <i>Batrachochytrium salamandrivorans</i> (Chapter 2.1.3.)	19
8.2.3. Infection with infectious haematopoietic necrosis virus (Chapter 2.3.4.)	19
8.2.4. Infection with viral haemorrhagic septicaemia virus (Chapter 2.3.10.)	20
8.3. Texts for Member comments	21
8.3.1. Infection with <i>Gyrodactylus salaris</i> (Chapter 2.3.3.).....	21
8.3.2. Infection with salmonid alphavirus (Chapter 2.3.6.)	21
8.4. Texts for Member information	22
8.4.1. Updating the introductory chapters for each host group: amphibians, crustaceans, fish and molluscs (Chapters 2.1.0, 2.2.0, 2.3.0. and 2.4.0, respectively)	22
8.4.2. The use of environmental DNA methods for aquatic animal disease surveillance	22

9. OIE AD HOC GROUP	22
9.1. Electronic ad hoc Group on Tilapia lake virus	22
9.2. Ad hoc Group on Susceptibility of mollusc species to infection with OIE listed diseases	22
10. OIE REFERENCE CENTRES OR CHANGE OF EXPERT	23
10.1. Evaluation of applications for OIE Reference Centres for Aquatic Animal Health issues or change of experts.....	23
10.2. Evaluation of annual reports from the OIE Reference Laboratories	23
10.3. Evaluation of the 5-year work plans from the OIE Collaborating Centres for disease of Aquatic Animals	24
11. OTHER ISSUES	24
12. DATE OF NEXT MEETING	24

1. WELCOME FROM THE DEPUTY DIRECTOR GENERAL

Dr Matthew Stone, Deputy Director General (International Standards and Science), welcomed the Specialist Commission and thanked both the members for taking time from their busy schedules to support the work of the OIE, and their employers and national governments.

Dr Stone provided a briefing on the OIE involvement in the COVID-19 international response led by the World Health Organization.

Dr Stone noted the draft 7th Strategic Plan had recently been circulated to Delegates, and he summarised the revisions to the OIE Headquarters organigram made in late 2019 as a result of the organisational assessment processes linked to the strategy development. Dr Stone briefed the Aquatic Animals Commission on initiatives in relation to Good Regulatory Practices, including expectations relating to regulatory stewardship, the completion of the design phase of the OIE Observatory, and the initiation of work on an on-line commenting system for standards development and review. He noted the intention to produce a clear articulation of the OIE Science System, building on work over recent years to more clearly describe process and performance management expectations of OIE Reference Centres, and committed to ongoing engagement with the Specialist Commissions during this work.

Finally, Dr Stone provided an update on the Specialist Performance Management System, focussing on the evaluation phase to be initiated in the second half of 2020 prior to the next elections for Specialist Commissions in 2021.

2. ADOPTED AGENDA

The adopted agenda of the meeting is presented as [the Table of Contents](#).

3. MEETING WITH THE DIRECTOR GENERAL

Dr Monique Eloit, OIE Director General, acknowledged the significant work being undertaken by the Aquatic Animals Commission and thanked the Commission for its ongoing commitment. Dr Eloit also thanked the members and the Secretariat for their work in finalising the OIE's Aquatic Animal Health Strategy. Dr Eloit considered this to be a very important strategy for the OIE that will provide a clear strategic vision to strengthen the efforts of OIE staff, Members, partners and donors to improve aquatic animal health and welfare worldwide.

4. COOPERATION WITH OTHER SPECIALIST COMMISSIONS

The Aquatic Animals Commission met with the Secretariats for the Terrestrial Animal Health Standards Commission (the Code Commission) and the Biological Standards Commission to share information and explore areas of common interest.

5. OIE AQUATIC ANIMAL HEALTH STRATEGY

The Aquatic Animals Commission continued its work to finalise the draft OIE Aquatic Animal Health Strategy (Aquatic Strategy).

The OIE Director General committed the OIE to developing a strategy for aquatic animal health at the 4th OIE Global Conference on Aquatic Animal Health in Chile (April 2019) and reiterated this commitment at the 87th General Session in May 2019.

Development of the Aquatic Strategy is an initiative led by the OIE with the support of the Aquatic Animals Commission. It recognises the growing importance of aquatic animal health and the need for a strategic approach to its management worldwide. The Aquatic Strategy supports the 7th OIE Strategic Plan and is aligned with the mandate of the OIE. It has been shaped heavily by inputs from the OIE Community to reflect the highest priority common needs of Members.

The views of Members and OIE experts were sought through a range of fora including the OIE General Session, Regional Commission Conferences, Global Conferences and technical items. Through these fora, the need for an Aquatic Strategy and clear themes for its objectives emerged.

With the objectives identified for the Aquatic Strategy, the next step was to seek feedback from the OIE Community on priority activities. In December 2019, the OIE conducted a consultation of the OIE Community including Members (OIE Delegates and relevant OIE Focal Points), regional and sub-regional offices, Reference Centres and other international partners.

The consultation sought views on four areas: i) what OIE activities are already working well, ii) what are the greatest opportunities, iii) what are the greatest threats to the growth of aquatic animal production over the next 5-10 years and iv) prioritisation of proposed activities within the strategy itself.

Responses provided valuable input to shape the strategy, confirmed the needs of Members and prioritised the activities to be included in the strategy.

February 2020 meeting

During its February 2020 meeting, the Aquatic Animals Commission met with relevant staff at OIE Headquarters to present the draft and invite feedback, which the Commission considered critical as the Aquatic Strategy is a transversal activity involving many OIE Departments in its implementation.

Following validation by Dr Eloit, it was intended that the OIE Aquatic Strategy be launched at the 88th General Session in May 2020. Alternative plans for launch of the strategy are to be determined.

6. WORK PLAN OF THE AQUATIC ANIMALS COMMISSION

Comments were received from Chile, New Caledonia and the EU.

The Aquatic Animals Commission reviewed comments received from Members and informed Members that it would address safe commodities by reviewing the structure of Article X.X.3 of all disease-specific chapters as presented in the Commission's work plan, which had been agreed at earlier meetings, under the item 'improvement of standards in Sections 8-11'. The Commission agreed to progress this work before February 2021.

The Commission considered a request to reprioritise the order of the remaining diseases for the assessment of susceptible species; however, it was agreed not to change the work plan as it was already well underway with the relevant *ad hoc* Groups.

7. OIE AQUATIC ANIMAL HEALTH CODE

The Aquatic Animals Commission noted that where amendments were of an editorial nature, no explanatory text has been provided in this report.

7.1. Texts to be proposed for adoption in May 2021

7.1.1. New draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X)

Comments were received from Argentina, Australia, Canada, Chile, Chinese Taipei, Cuba, Japan, New Caledonia, New Zealand, Nicaragua, Norway, the USA, the EU and AU-IBAR.

Background

The new draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X) is the second new chapter to be developed as part of the ongoing revision of Section 4, Disease Prevention and Control. The draft chapter has been circulated three times for comment between September 2018 and September 2019.

At the September 2019 meeting, the Aquatic Animals Commission revised the chapter in response to two important issues raised by Members: following and biosecurity at the compartment level.

Previous Commission reports where this item was discussed:

September 2018 report (Item 2.9, page 61); February 2019 report (Item 2.1, page 103); September 2019 report (Item 6.1, pages 21 and 33).

February 2020 meeting

General comments

The Commission noted that comments received supported the draft chapter and that the majority of comments were editorial in nature.

The Commission reminded Members that Glossary terms are not italicised in titles.

Article 4.X.3 Introduction

For consistency with other parts of the chapter, the Commission agreed to add ‘welfare’ to the opening sentence of the first paragraph.

The Commission agreed to add ‘may’ in the last paragraph, ‘The benefits may...’, noting that implementation of biosecurity will not necessarily result in improved market access.

Article 4.X.4 General principles

The Commission did not agree to amend the Glossary definition of ‘biosecurity’ in line with the OIE *Terrestrial Animal Health Code (Terrestrial Code)*. The Commission noted that the terms ‘infection’, ‘infestation’ and ‘disease’ are used differently in the Codes.

In paragraph 1, the Commission agreed to move ‘planning’ to the beginning of the second sentence of the first paragraph to emphasise that this component is an important first step for biosecurity.

In paragraph 1, the Commission did not agree with a request to delete the reference to ‘within an aquaculture establishment’ because the scope of this chapter is specific to biosecurity in aquaculture establishments.

The Commission inserted a new point 5) to address signage, as it considered this an important tool to raise awareness and understanding of certain biosecurity measures.

For point 1, regarding potential pathways, the Commission agreed to delete the reference to Article 4.X.5, noting that this cross reference was not correct as it refers to categories of aquaculture production systems and not pathways.

The Commission agreed with a comment that it is important to highlight that visitors to aquaculture establishments are briefed and supervised to ensure compliance with the biosecurity plan. However, the Commission considered that this issue was best addressed by the addition of a new point 7, Personnel and visitors, in Article 4.X.6, Transmission pathways and mitigation measures.

The Commission clarified that third-party audits would be undertaken by, or on behalf of, customers or regulators as noted in point 7.

Article 4.X.5 Categories of aquaculture production systems

The Commission agreed to add ‘system’ to all the category titles.

The Commission did not agree to add ‘troughs’ as examples of semi-closed systems because ‘trough’ is not a commonly used term. It also noted that enclosed floating pens are considered as semi-closed systems because there is partial control over water entering and exiting the system.

For closed systems, the Commission agreed to move the last sentence, ‘Environmental conditions can also be controlled’, to the beginning of the paragraph to improve clarity.

Article 4.X.5 bis Area management

The Commission did not agree with a comment to include a Glossary definition for ‘area management’ as it considered the context of ‘area management’ was clear; in addition, the term is used infrequently throughout the *Aquatic Code*.

The Commission did not agree to remove the term ‘area’ throughout the text of this article and replace it with ‘zone’, noting that aquaculture establishments may be in defined geographic areas (e.g. bays) rather than ‘zones’ with a specific health status.

The Commission agreed to add ‘coordination of’ in the last sentence of the article to highlight that cooperation in the implementation of biosecurity measures is required among aquaculture establishments in shared water bodies.

The Commission considered to highlight the importance of ongoing communication between aquaculture establishments but agreed that this was not necessary as ‘coordination’ among the aquaculture establishments would include and implies such communication.

Article 4.X.6 Transmission pathways and mitigation measures

1. Aquatic animals

For point 1, regarding transmission risks associated with aquatic animals, the Commission did not agree with a comment to elaborate the requirements for exporting countries. The Commission considered that this issue was outside the scope of this chapter, which is restricted to the biosecurity of aquaculture establishments. The Commission agreed that the wording of point b) was not clear and could be read as contradictory to point a) and amended the wording to state that if aquatic animals of unknown status are introduced, they should be placed into quarantine.

The Commission did not agree that conditions in quarantine need to be described as ‘conducive to clinical expression’. It considered that the glossary definition for quarantine is sufficient for the purposes of this chapter. However, the Commission acknowledged that further guidance on quarantine could be beneficial, and it agreed to consider this issue in the context of its work plan.

For point d), the Commission did not agree with a proposal to include ‘stress’, noting that the focus of this point is exposure to pathogenic agents.

For point e), the Commission agreed to emphasise that movements of aquatic animals between different populations should only be done with the view to maintaining the highest possible health status of the populations and amended the text accordingly.

For point h), the Commission acknowledged a comment that Competent Authorities must follow the OIE guidance on notification of suspicion of disease but did not agree to delete the reference to local requirements in the text. The Commission explained that local requirements provide the legal basis for reporting to the Competent Authority and may exceed the reporting obligations of the Competent Authority to the OIE. The Commission did not agree to add ‘by the Aquatic Animal Health Services under the direction of the Competent Authority’ at the end of this point, noting that such investigations and the diagnosis of the cause of mortality may be carried out at the farm level in some circumstances.

For the last sentence in i), the Commission did not agree with a proposal to state that fallowing should be coordinated between aquaculture establishments that are subject to an area management agreement, as it considered that it is possible to coordinate synchronous fallowing on farms in a shared water body without a formal agreement.

For point j), the Commission did not agree with a comment to replace ‘infection’ with ‘pathogenic agents’ because the focus of this point is on infection cycles.

The Commission did not agree to add a new point k) to cover animal movement records because record keeping is not a mitigation measure. It also noted that record keeping is covered in point 2 b) of Article 4.X.8, Biosecurity plan development.

2. Aquatic animal products and aquatic animal waste

For point 2, regarding transmission risks associated with aquatic animal products and aquatic animal waste, the Commission agreed to delete ‘aquatic animals’ in the last sentence of the first paragraph to improve readability.

In the second paragraph, the Commission agreed to delete the second last sentence concerning high risk waste as it considered this to be a repetition.

For point b), the Commission did not agree to add ‘and adopting biosecurity measures’ because the adoption of biosecurity measures is implicit in the implementation of a biosecurity plan.

In point c), the Commission agreed to replace 'systems' with 'procedures' in the first sentence and to align the last sentence with the wording used in the previous point b). It did not agree to add 'removal' because 'collection' includes removal, whether it is removal from pens or from the aquaculture establishment.

3. Water

For point 3, regarding transmission risks associated with water, the Commission did not agree to change the phrase 'risk of introduction of pathogenic agents into, spread within, and release from aquaculture establishments' in the first paragraph, noting that this terminology is used throughout the chapter.

The Commission did not agree to delete the last sentence of b) 'The level of treatment required will depend on the identified risks.' because it provides important context. It added 'type of' to the sentence to emphasise that the selection of treatment method will depend on the identified risks.

The Commission agreed to add 'and filtered waste' to point c) because this type of waste is not specified elsewhere in the chapter. It also agreed to add 'type' to the last sentence because the selection of a disinfection method(s) will be determined by its effectiveness against the specific pathogenic agent. It also agreed to delete the word 'holding', noting that it was superfluous.

In response to comments, a new point f) was added to address measures to mitigate the risk of water entering the aquaculture establishment via the transport of aquatic animals. The Commission highlighted that further guidance on management of transport water is provided in Chapter 5.5, Control of aquatic animal health risks associated with transport of aquatic animals.

4. Feed

The Commission did not agree to add a new point to emphasise the importance of appropriate storage and management of feed on site and practices to mitigate the risk of contamination of feed, as it considered that this point was addressed in sufficient detail in the proposed text.

5. Fomites

For point 5, regarding transmission risks associated with fomites, the Commission agreed to amend the text to include fomites moved within, and from, the aquaculture establishment.

The Commission did not agree to specify that disease risks of fomites that have been in direct contact with aquatic animals should receive special attention, because risk assessment would address all levels of disease risk.

6. Vectors

For point 6, regarding transmission risks associated with vectors, the Commission agreed to replace 'risk' with 'likelihood' in the first paragraph. Similar amendments were made throughout the chapter, where appropriate, to reflect the intended meaning.

For point a), the Commission agreed to make an editorial change to improve readability. It did not agree to delete 'or disinfection' in a) i) because only physical mitigation measures are addressed under this point.

For point b), the Commission did not agree to add a sentence that pest control should be carried out in an ethical and environmentally friendly manner because such guidance is not within the scope of the chapter.

7. Personnel and visitors

In response to a comment, the Commission added a new point 7, Personnel and visitors, recommending that visitors to aquaculture establishments are briefed and supervised to ensure compliance with the biosecurity plan.

The Commission agreed that signage was important to raise awareness and concluded that it was best addressed in Article 4.X.4, General principles, rather than in Article 4.X.6, Transmission pathways and mitigation measures.

Article 4.X.7 Risk analysis

The Commission noted a request to include the potential establishment of pathogenic agents in the environment in the descriptors for consequence in Table 2. However, the Commission declined to make the suggested change because the chapter concerns biosecurity for aquaculture establishments, and thus the assessment of risk for pathogen establishment in the environment is outside of its scope.

The Commission did not agree to delete the reference to trade in Table 2 noting that, for the purpose of this chapter, trade described at the level of the aquaculture establishment is relevant.

In Table 3, the Commission did not agree to change the title to ‘Matrix for assessing risk’ as it considered it to be clear and accurate as written. It did, however, agree to replace ‘medium’ with ‘low’ under the insignificant consequence and certain likelihood combination as it agreed this was a more appropriate risk estimate.

Under Step 3, the Commission did not agree to delete ‘cost-effective’ from the first paragraph because it considered this an important part of prioritising mitigation measures.

Article 4.X.8 Biosecurity plan development

For point 1a), regarding the development of a biosecurity plan, the Commission agreed to add ‘scope’ because it is an important part of the biosecurity plan. It also agreed that the plan should include information on access points to the aquaculture establishment and added text to address this in point b).

For point 2, the Commission agreed to add ‘quarantine measures’ in the examples of documentation required in b). The Commission did not agree to include ‘regulatory requirements’ because this was covered elsewhere in the chapter.

In point c), the Commission did not agree to specify that the reporting requirements should be limited to reporting to Competent Authorities, because procedures could include reporting to the management of the aquaculture establishment or to private aquatic animal health professionals.

In point c), the Commission did not agree to add ‘contingency planning’ to the title because contingency planning is outside the scope of this chapter.

The revised new Chapter 4.X, Biosecurity for Aquaculture Establishments, is presented in [Annex 2](#) for Member comments.

EU comment

The EU thanks the OIE and supports the revised new Chapter 4.X contained in Annex 2.

The adoption of the chapter has been postponed until the 89th General Session in May 2021. As the chapter has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

7.1.2. Listing of infection with decapod iridescent virus 1 (DIV1) – Revised Article 1.3.3 of Chapter 1.3

Comments were received from Chinese Taipei, New Caledonia, Nicaragua and the EU.

Background

The Commission, at its February 2019 meeting, assessed infection with shrimp haemocyte iridescent virus (SHIV) against the criteria for listing aquatic animal diseases in Article 1.2.2 and agreed that infection with SHIV meets the OIE criteria for listing and should be added to Article 1.3.3, Diseases of crustaceans listed by the OIE. At this meeting the name was changed to 'Infection with decapod iridescent virus 1 (DIV1)' in accordance with the classification of the pathogenic agent in the database of the International Committee of Taxonomy of Viruses (ICTV).

The proposal to list infection with DIV1 has been circulated three times for comments, and each time the assessment against the criteria for listing for information was included.

Previous Commission reports where this item was discussed:

February 2019 report (Item 3.1.1, page 113); September 2019 report (Item 6.2, page 45).

February 2020 meeting

The Commission noted that all comments received supported the listing of infection with DIV1.

In response to a request to consider introducing acronyms in the list of diseases, the Commission noted that this is not the convention for Chapter 1.3 but emphasised that acronyms and common disease names are used in the disease-specific chapters.

The revised Article 1.3.3 of Chapter 1.3, Diseases listed by the OIE, is presented in [Annex 3](#) for Member comments (see also Item 7.3.4.).

EU comment

The EU thanks the OIE and supports the revised article 1.3.3 of Chapter 1.3. presented in Annex 3.

Note: The adoption has been postponed until the 89th General Session in May 2021. As the Article has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

7.1.3. Model Article 10.X.13 for the fish disease-specific Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 for Chapter 10.4)

Comments were received from Australia, Chile, Chinese Taipei and the EU.

Background

Revision of Article 10.X.13, Importation of disinfected eggs for aquaculture from a country, zone or compartment not declared free from infection with pathogenic agent X, for the fish disease-specific Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 for Chapter 10.4), was initiated by the

Aquatic Animals Commission in February 2019 in response to requests to clarify the intended purpose of this article. The model article has been circulated twice for comments.

Previous Commission reports where this item was discussed:

September 2019 report (Item 6.3, page 47); February 2020 meeting.

The Commission noted that comments received supported the proposed amendments and included some amendments of an editorial nature.

The Commission agreed to include in the first paragraph a cross reference to Chapter 4.4, Recommendation for surface disinfection of salmonid eggs, to provide guidance to Members on disinfection protocols.

For point 1, the Commission did not agree to include a recommendation for Competent Authorities of importing countries to gain assurances from the Competent Authority of the exporting country because it considered this to be implicit in the current text.

The Commission did not agree to refer to the protocol in point 1 a) of Article 4.4.2 (of Chapter 4.4) because other protocols may be equally effective. It also did not agree to include text referring to Article 4.4.1) (of Chapter 4.4) in point c) as it considered this unnecessary detail and the reference to Chapter 4.4 in the leading text for point 1) was sufficient.

The Commission amended the first sentence of point 2 to clarify that the Competent Authority of the importing country cannot itself apply measures pre-import, as acknowledged in Article 3.

For point 2, the Commission did not agree to include procedures for species other than for salmonids. It reiterated that it had asked Members on several occasions to provide protocols for other species, but to date without response.

The Commission did not agree to remove 'including' in point 2. It noted its use allows for additional measures than those listed, and removing it would limit the options. The Commission decided that disinfection upon arrival should not be a requirement, but a post-importation measure that a Member should consider.

The Commission amended point 3 to restrict the application of international health certification to point 2 a) and b), because internal measures also given in point 2 are not relevant to international certification.

The revised Model Article 10.X.13 for the fish disease-specific Chapters 10.5, 10.6 and 10.10 (and Article 10.4.17 for Chapter 10.4) is presented in [Annex 4](#) for Member comments.

EU comment

The EU thanks the OIE and supports Annex 4.

Note: The adoption has been postponed until the 89th General Session in May 2021. As the Article has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

7.1.4. Article 10.9.2 of Chapter 10.9 Infection with spring viraemia of carp virus

Comments were received from Canada, China (People's Rep. of), Chinese Taipei and the USA.

Background

The revised list of susceptible species in Article 10.9.2 of Chapter 10.9, Infection with spring viraemia of carp virus (SVCV), was adopted at the 87th General Session in May 2019. However, in

light of new scientific evidence on the susceptibility of zebrafish to infection with SVCV, the Aquatic Animals Commission reviewed the previous assessment for this species and agreed that zebrafish did meet the criteria for listing as a susceptible species and should, therefore, be included in Article 10.9.2. This proposal was circulated for comment in the Commission's September 2019 report.

February 2020 meeting

The Commission noted that Members supported zebrafish (*Danio rerio*) being included in the list of susceptible species in Article 10.9.2.

A request was made to list yellow Perch (*Perca flavescens*) and sockeye salmon (*Oncorhynchus nerka*) as susceptible species for infection with SVCV in Article 10.9.2 based on evidence in the study by Emmenegger *et al.*, 2016. In response, the Commission reminded Members that the assessments for these two species conducted by the *ad hoc* Group on Susceptibility of fish species to OIE listed diseases in November 2017 had considered this paper. The *ad hoc* Group had concluded that these species did not meet the criteria for susceptibility because the route of experimental infection described in the study was invasive. The Commission reiterated its previous decision not to list these two species as susceptible.

The Commission informed Members that if they wish to propose the listing of additional species, they are encouraged to read relevant *ad hoc* Group reports (<https://www.oie.int/en/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/>), and to submit new scientific evidence in support of their proposal.

Reference: Emmenegger, A. J., Sanders, G. E., Conway, C. M., Binkowski, F. P., Winton J. R. and Kurath, G. (2016). Experimental infection of six North American fish species with the North Carolina strain of spring Viremia of Carp Virus. *Aquaculture*, 450, 273–282.

The revised Article 10.9.2 of Chapter 10.9, Infection with spring viraemia of carp virus, is presented as [Annex 5](#) for Member comments. The adoption has been postponed until the 89th General Session in May 2021. As the Article has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

EU comment

The EU thanks the OIE and supports the changes contained in Annex 5.

7.1.5. Article 10.10.2 of Chapter 10.10 Infection with viral haemorrhagic septicaemia virus

Comments were received from Canada, Korea (Rep. of), Thailand, the USA and the EU.

Background

The *ad hoc* Group on Susceptibility of fish species to OIE listed diseases had applied the criteria for listing species as susceptible to infection with viral haemorrhagic septicaemia virus (VHSV) in accordance with Chapter 1.5, Criteria for listing species as susceptible to infection with a specific pathogen (report available at <https://www.oie.int/en/standard-setting/specialists-commissions-working-ad-hoc-groups/ad-hoc-groups-reports/>). The assessments were reviewed by the Commission at its September 2019 meeting and the amended list of susceptible species in Article 10.10.2 was circulated for comment for the first time in the Commission's September 2019 report.

Previous Commission reports where this item was discussed:

September 2019 report (Item 6.4, page 49).

February 2020 meeting

The Commission confirmed that zebra fish (*Danio rerio*) does meet the criteria for listing as susceptible because infection has been demonstrated by a natural route of transmission

(immersion) by Cho *et al.* (2019); however, this study had been inadvertently left out of the *ad hoc* Group's report. Species can meet the criteria for listing as susceptible based on experimental studies but in such cases, consideration needs to be given to whether experimental procedures (e.g. injection, infective load) mimic natural pathways for disease transmission.

In response to a comment about the interpretation of the application of the criteria for listing species as susceptible, the Commission acknowledged that, although the interpretation of evidence required to assess a species as susceptible had not changed, it agreed that it had not been sufficiently described in previous reports. The Commission emphasised that, as noted in the September 2019 report of the *ad hoc* Group on Susceptibility of fish species to infection with OIE listed diseases, if only a single study provided evidence for susceptibility of a species, some form of corroborating evidence was also required to satisfy the criteria for susceptibility. Corroborating evidence may be found either within the scientific reference paper or in other papers.

Several Members commented on the listing of VHSV genotypes in the table of susceptible species in Article 10.10.2. The Commission agreed to remove genotypes from the table because knowledge of species susceptibility by genotypes was incomplete and the information was not relevant for trade purposes. Any differentiation of trade measures based on genotype would require that the case for VHSV strain differentiation be assessed by the Commission using the approach that the Commission had previously applied to ISAV.

However, the Commission agreed that information on VHSV genotypes should be included in the VHSV chapter of the *Aquatic Manual* in the list of susceptible species (2.2.1) and the list of species with incomplete evidence for susceptibility (2.2.2), as this is valuable epidemiological information. The Commission noted that the *Aquatic Manual* Chapter 2.3.10, Viral haemorrhagic septicaemia, is currently being reformatted into the new template and the list of genotypes will be checked in accordance with this proposal by the OIE Secretariat.

The Commission decided to clearly mark when there is a lack of information about genotype for a susceptible species to avoid confusion.

The Commission requested the OIE Secretariat to review the list of references in the *ad hoc* Group report to correct errors, make necessary amendments and update the version on the OIE website.

A Member requested that brook trout (*Salvelinus fontinalis*) and burbot (*Lota lota*) be considered for listing as species susceptible to VHSV based on unpublished information. The Commission reiterated that i) the *ad hoc* Group can only consider published information and ii) these two species had been assessed by the *ad hoc* Group and found not to meet the criteria for susceptibility. The Commission encouraged Members to provide any new scientific evidence to the Commission for its review.

The Commission did not agree with a request to add 'olive flounder' as a common name for bastard halibut (*Paralichthys olivaceus*), and reminded Members that it had agreed to refer to common names of fish species in line with FAOTERM (<http://www.fao.org/faoterm/collection/faoterm/en/>) and scientific fish names in line with Fishbase (<https://www.fishbase.se/search.php>).

The Commission amended the scientific name of brown bullhead from '*Ictalurus nebulosus*' to '*Ameiurus nebulosus*' in line with the naming in Fishbase.

Reference: Cho, S. Y., Protzman, R. A., Kim, Y. O., Vaidya, B., Oh, M. J., Kwon, J. and Kim, D. (2019). Elucidation of mechanism for host response to VHSV infection at varying temperatures *in vitro* and *in vivo* through proteomic analysis. *Fish and Shellfish Immunology*, 88, 244–253.

The revised Article 10.10.2 of Chapter 10.10, Infection with viral haemorrhagic septicaemia virus, is presented as [Annex 6](#) for Member comments.

EU comment**The EU thanks the OIE and supports Annex 6.**

Note: The adoption has been postponed until the 89th General Session in May 2021. As the Article has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

7.1.6. Glossary definitions for ‘Aquatic animal waste’ and ‘aquatic animal products’

Comments were received from Chile, Canada, New Zealand, the EU and AU-IBAR.

Background

At its September 2019 meeting, the Aquatic Animals Commission proposed a new Glossary definition for ‘aquatic animal waste’ given that the term is used extensively in the new draft chapter on Biosecurity for aquaculture establishments (Chapter 4.X) as well as in Chapter 4.7, Handling, disposal and treatment of aquatic animal waste. The new Glossary definition was circulated for comment in the Commission’s September 2019 report.

Previous Commission reports where this item was discussed:

September 2019 report (Item 6.7, page 79).

February 2020 meeting

The Commission considered comments and amended the definition of ‘aquatic animal waste’ to make the distinction between aquatic animal products and aquatic animal waste, and to distinguish aquatic animal waste from aquatic animal by-products. As a consequence of these amendments, the Commission also made changes to the definition of ‘aquatic animal products’ to align it with the new definition of ‘aquatic animal waste’.

The Commission noted that, as per convention, once the new Glossary definition for ‘aquatic animal waste’ is adopted, the definition for ‘aquatic animal waste’ in Article 4.7.3 will be deleted.

The Commission also reviewed the use of the term ‘waste’ throughout the *Aquatic Code* and proposed that once the new definition for ‘aquatic animal waste’ is adopted, the term ‘waste’ will be amended to the italicised term ‘aquatic animal waste’, where relevant, to reflect the defined term.

EU comment**There is an error with the numbers of the annexes. The proposed amendments of the term ‘waste’ to ‘aquatic animal waste’ in the Aquatic Code are in Annex 8.**

The revised Glossary definitions for ‘aquatic animal waste’ and ‘aquatic animal products’ are presented in [Annex 7](#) for Member comments.

EU comment**The EU thanks the OIE and supports the revised Glossary definitions in annex 7 and 8 (see EU comment above).**

Note: The adoption has been postponed until the 89th General Session in May 2021. As the Glossary definitions have already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

7.2. Texts for Member comments

7.2.1. Revised Glossary definition for ‘vector’

In response to a request from the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases, the Commission amended the Glossary definition of ‘vector’ to make it clear that vectors for a specified infectious agent cannot be listed as a susceptible species for the same agent.

The revised Glossary definition for ‘vector’ is presented in [Annex 8](#) for Member comments.

EU comment

There is an error with the numbers of the annexes. The revised definition of ‘vector’ is in annex 7.

Annex 8 contains the proposed amendments of the term ‘waste’ to ‘aquatic animal waste’ in the Aquatic Code.

7.2.2. Approaches for determining periods required to demonstrate disease freedom

Background

A discussion paper on approaches for determining periods required to demonstrate disease freedom, developed by the Aquatic Animals Commission, was first circulated for comments in the Commission’s September 2018 report. The Commission considered comments received and circulated a revised discussion paper in its September 2019 report.

Previous Commission reports where this item was discussed:

September 2018 report (Item 2.10, page 71); September 2019 report (Item 6.6, page 55).

February 2020 meeting

Comments were received from Australia, Canada, Chile, China (People’s Rep. of), Japan, Nicaragua, New Zealand, Norway, Thailand, the USA and the EU.

The Commission acknowledged the constructive comments submitted on the discussion paper and concluded that the paper had served its purpose. Based on the comments, the Commission focused on revising model Articles X.X.4, X.X.5 and X.X.6 in the disease-specific chapters of the *Aquatic Code* on disease freedom and Chapter 1.4.

Model articles for disease-specific chapters were drafted to cover issues related to establishing disease freedom at country, zone and compartment level, e.g. vaccination and basic biosecurity conditions, which will be cross-referenced in relevant articles in Chapter 1.4.

The structure of existing model Articles X.X.4 to X.X.6 will be retained but revisions are proposed. The absence of susceptible species will not be available as a pathway for disease freedom for pathogens with a broad host range (currently this pathway is not available for infection with *Aphanomyces invadans* (Epizootic ulcerative syndrome) and infection with viral haemorrhagic septicaemia virus [VHSV]). Separate articles have been drafted for establishing disease freedom for a zone and compartment and a new article on restoring freedom at compartment level has been included. The Commission decided that targeted surveillance is always required to establish freedom for a compartment (historic freedom and absence of susceptible species are not appropriate pathways for disease freedom at compartment level).

Chapter 1.4 will be revised to focus on supporting provisions of the disease-specific *Aquatic Code* chapters. The current Article 1.4.6 will be expanded into multiple articles and include criteria and approaches set out in the discussion paper (e.g. guidance on surveillance to achieve disease freedom). The generic information on surveillance and examples of surveillance systems in the current Chapter 1.4 will be considerably shortened or removed.

Model Articles X.X.4, X.X.5 and X.X.6 for the disease-specific chapters of the *Aquatic Code* are presented in [Annex 9](#) for Member comments.

EU comment

The EU in general supports the changes in Annex 9. Several EU comments have been included.

7.3. Texts for Member information

7.3.1. De-listing of infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) – Revised Article 1.3.3 of Chapter 1.3

The Commission had received a request from a Member to remove infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV) from the list of diseases in Article 1.3.3 of Chapter 1.3, Diseases listed by the OIE. The Commission agreed to consider the issue further at its September 2020 meeting. It requested that Members provide any available information relevant to the Criteria for Listing Aquatic Animal Diseases; specifically listing criteria 4b) and 4c) of Article 1.2.2 (consequences for cultured or wild aquatic animals respectively).

The Commission wished to advise Members that any recommendation to de-list IHHNV would be provided to Members for consideration – together with an assessment against the listing criteria.

7.3.2. New draft chapters on emergency disease preparedness and disease outbreak management

The Commission agreed to commence work on two new chapters for the revised Section 4, Disease Prevention and Control: i) Emergency disease preparedness and ii) Disease outbreak management. The Commission agreed to define the article structure for both chapters, given how closely the two chapters are linked, and to review this work at its September 2020 meeting.

7.3.3. Infection with Carp edema virus (CEV)

The Commission reviewed scientific information on infection with carp edema virus (CEV) given that it has been reported recently in several countries in the Asia-Pacific region and appears to be extending its geographic range. The Commission noted that the disease was listed as a notifiable disease by the Network of Aquaculture Centres in Asia-Pacific (NACA) in 2017 and that it has been reported to significantly impact carp production, which is the largest fish production in the world.

Based on available scientific information, the Commission agreed that infection with CEV meets the OIE definition of an ‘emerging disease’ and, as such, Members should report it in accordance with Article 1.1.4 of the *Aquatic Code*. The Commission also encouraged Members to investigate mortality and morbidity events linked to this disease, emphasising that a better understanding of the virus is essential for efforts to control its possible spread. Members are encouraged to submit information on their experiences with infection with CEV and its impacts to the Commission, which will review the situation at its September 2020 meeting.

7.3.4. Assessment of infection with decapod iridescent virus 1 (DIV1) for listing in Chapter 1.3 of the *Aquatic Code*

The Commission accepted most of the comments received on the assessment of infection with DIV1 against the criteria for listing aquatic animal diseases in Article 1.2.2, Criteria for listing aquatic animal diseases, to improve clarity

The revised assessment of infection with DIV1 is presented in [Annex 10](#) for Member information (see also Item 7.1.2.).

8. OIE MANUAL OF DIAGNOSTIC TESTS FOR AQUATIC ANIMALS

8.1. Status of *Aquatic Manual* revisions

The Aquatic Animals Commission examined the status of chapters that had previously been identified for substantive revision. These chapters include those in Section 2.3, Diseases of Fish, and a new chapter on Infection with *Batrachochytrium salamandrivorans*. The Commission agreed to a target of circulating first drafts of the remaining fish disease chapters, which are revised using the new template, for review at the September 2020 meeting.

The Commission reiterated the decision to no longer revise the chapters on delisted diseases and to remove them from the *Aquatic Manual* following the General Session in May 2021. The Commission noted that it was important to focus available resources on maintaining the quality of chapters for listed diseases. The chapters for delisted diseases would remain available in previously published versions of the *Aquatic Manual*.

8.2. Texts to be proposed for adoption in May 2021

Horizontal amendments

The Aquatic Animals Commission agreed to a proposal to add the word ‘apparently’ in the title of Table 4.1, OIE recommended diagnostic methods and their level of validation for surveillance of healthy animals and investigation of clinically affected animals, before the words ‘healthy animals’. This amendment will be made horizontally to all *Aquatic Manual* chapters and to the template.

The Commission did not agree to combine test methods in the rows of Table 4.1, for example to combine cell culture and a molecular or immunological method in the same row. The Commission reminded Members that test methods are listed separately in Table 4.1, and that combinations of test methods, necessary to define a suspect or confirmed case, are given in Section 6, Corroborative diagnostic criteria.

In response to a number of comments regarding the use of abbreviations, the Commission reiterated that in the *Aquatic Manual*, the abbreviation ‘RT-PCR’ is reserved for reverse-transcription polymerase chain reaction methods; ‘real-time PCR’ is always stated in full. The term ‘conventional PCR’ is used throughout the *Aquatic Manual*, including in Table 4.1 and the title of Section 4.4.2.

The Commission accepted a request to delete ‘serological’ from the title of Section 4.10, Other serological methods, as the other methods should not be limited to serological ones. This change will be made horizontally to all the *Aquatic Manual* chapters and to the template.

The Commission did not agree with a number of proposed changes to the text in Section 6, Corroborative diagnostic criteria. The text in this Section is given in the chapter template and is standard throughout all the chapters.

The Commission agreed with a comment to add an explanation of the purpose of Table 6.1, Diagnostic performance of tests recommended for surveillance or diagnosis, given in Section 6.3, Diagnostic sensitivity and specificity for diagnostic tests. This change will be made horizontally to all the *Aquatic Manual* chapters and to the template. The Commission reiterated that the information included in the table

is important for the interpretation of test results; where there is no information available to complete the table (due to a lack of estimates for diagnostic sensitivity and specificity), this will be stated in this section.

8.2.1. Infection with spring viraemia of carp virus (Chapter 2.3.9)

Comments were received from Australia, Canada, Chinese Taipei, China (People's Rep. of), Korea (Rep. of), Singapore, Thailand, the EU and AU-IBAR.

The Aquatic Animals Commission did not agree with a comment proposing to delete the fire belly newt (*Cynops* spp.) and the white leg shrimp (*Penaeus vannamei*), from Section 2.2.2, Species with incomplete evidence for susceptibility, because these are not finfish species. The Commission noted that the *ad hoc* Group on susceptibility of fish species to infection with OIE listed diseases had found that these species met the criteria for inclusion in this section. The Commission stressed that the purpose of this Section is to provide information that may be relevant to risk assessment and to highlight issues that may warrant further research.

The Commission did not agree with the proposal to move rainbow trout (*Oncorhynchus mykiss*) from Section 2.2.2, Species with incomplete evidence for susceptibility, to Section 2.2.1, Susceptible host species, as the Member had not provided any new evidence that would justify a re-consideration of the *ad hoc* Group's conclusions.

In reply to a Member comment to add more recent information to Section 2.4.6, Disinfection of eggs and larvae, the Commission confirmed that the current reference is valid and that it was not aware of any more recent scientific studies on this matter.

In response to a request to expand the information given in Section 3.5.3, Fixed samples for histopathology, immunohistochemistry or in-situ hybridisation, the Commission indicated that generic information on fixation techniques is given in Chapter 3.2.0, General information. There was no rationale given for repeating the information in this chapter or for providing guidance specifically relevant to spring viraemia of carp virus.

A Member proposed adding a sentence to Section 3.6, Pooling of samples, on the need to consider the impact of pooling on decisions related to trade. The Commission noted that as the *Aquatic Manual* principally provides diagnostic test methods, it is not the appropriate text for provisions on trade.

For Table 4.1, OIE recommended diagnostic methods and their level of validation for surveillance of healthy animals and investigation of clinically affected animals, a Member proposed that the level of validation noted for cell culture should be raised from 1 to 3 because it has been used for many years. The Commission noted that levels of validation are based on studies published in peer-reviewed journals and in accordance with the OIE Validation Pathway given in Chapter 1.1.2, Principles and methods of validation of diagnostic assays for infectious diseases.

The Commission did not agree to add a sentence to Section 4.3, Cell or artificial media culture for isolation, stating that a test can be declared negative if no cytopathic effect (CPE) occurs, because of the risk of false positive results from unclear or atypical CPE.

The Commission did not agree to include a paragraph in Section 5, Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations, indicating that the tests described in the chapter are insufficient to achieve freedom. Again, the *Aquatic Manual* is not the appropriate text for such statements.

The revised Chapter 2.3.9, Infection with spring viraemia of carp virus, is presented in [Annex 11](#) for Member comments. The adoption of the revised chapter has been postponed until the 89th General Session in May 2021. As the revised chapter has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

EU comment

The EU thanks the OIE and in general supports Annex 11. One EU comment has been included in the body of the text of Annex 11.

8.2.2. Infection with *Batrachochytrium salamandrivorans* (Chapter 2.1.3)

Comments were received from Australia, Canada, China (People's Rep. of), Thailand, the EU and AU-IBAR.

A number of minor editorial comments were received, and the chapter was amended accordingly.

In Table 4.1, OIE recommended diagnostic methods and their level of validation for surveillance of healthy animals and investigation of clinically affected animals, the Aquatic Animals Commission agreed to delete the lateral flow assay from column A, Surveillance of apparently healthy animals, and to lower its rating in column B, Presumptive diagnosis of clinically affected animals, because of the method's low specificity. As a consequence, the method was removed from Section 6.1.1, Definition of suspect case in apparently healthy animals.

The revised Chapter 2.1.3, Infection with *Batrachochytrium salamandrivorans*, is presented in [Annex 12](#) for Member comments. The adoption of the revised chapter has been postponed until the 89th General Session in May 2021. As the revised chapter has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

EU comment

The EU thanks the OIE and in general supports Annex 12. We have included an EU comment.

8.2.3. Infection with infectious haematopoietic necrosis virus (Chapter 2.3.4)

Comments were received from Canada, China (People's Rep. of), Korea (Rep. of), Thailand, the EU and AU-IBAR.

To ensure consistency, the Aquatic Animals Commission reviewed this chapter together with Chapter 2.3.10, Infection with viral haemorrhagic septicaemia virus, because of the similarities between the two chapters.

The Commission agreed to extend the geographical range of infection with infectious haematopoietic necrosis virus (IHNV) to include Africa and to add the published reference to the chapter.

The Commission modified a proposed amendment to Section 2.3.4, Modes of transmission and life cycle, to clearly state that there is insufficient evidence to demonstrate true vertical transmission of IHNV, and to make the role of disinfection in preventing transmission more explicit.

A proposal to add the words 'of the *Aquatic Manual*' after '2.4' in the sentence "This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected." was rejected as the Sections referred to are within the chapter itself.

In Section 3.5, Preservation of samples for submission, a Member requested deletion of a reference to Chapter 2.3.0, General information, stating that the chapter does not provide details of viral sample preservation. The Commission noted that Section 2.2, Virological examination, of Chapter 2.3.0 describes transportation and antibiotic treatment of samples for virological examination, and therefore concluded that retaining reference to Chapter 2.3.0 is appropriate.

The Commission accepted the proposal to add the standard text to Section 3.6, Pooling of samples. The Commission did not agree however, to include a sentence on the need to consider the impact of pooling on decisions related to trade. The Commission reaffirmed that as the *Aquatic Manual* principally provides diagnostic test methods, it is not the appropriate text for provisions on trade.

The Commission reviewed and amended or added some of the levels of validation given in Table 4.1, OIE recommended diagnostic methods and their level of validation for surveillance of healthy animals and investigation of clinically affected animals.

The revised Chapter 2.3.4, Infection with infectious haematopoietic necrosis virus, is presented in [Annex 13](#) for Member comments. The adoption of the revised chapter has been postponed until the 89th General Session in May 2021. As the revised chapter has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

EU comment

The EU thanks the OIE and in general supports the changes contained in Annex 13. One comment has been included in section 2.3.4.

8.2.4. Infection with viral haemorrhagic septicaemia virus (Chapter 2.3.10)

The chapter had been updated with the assistance of all three OIE Reference Laboratories for VHS. Comments were received from Canada, China (People's Rep. of), Korea (Rep. of), Thailand, the EU and AU-IBAR.

To ensure consistency, the Aquatic Animals Commission reviewed this chapter together with Chapter 2.3.4, Infection with infectious haematopoietic necrosis virus, because of the similarities between the two chapters.

A Member proposed editing Section 2.2.1, Susceptible host species, to note susceptibility of *Clupea harengus* (Atlantic herring) to genotype IVa (in addition to genotype Ib and III). After reviewing the scientific information, the Commission concluded that it did not agree with the proposal as there is incomplete evidence for susceptibility for *C. harengus* to the genotype IVa of infection with viral haemorrhagic septicaemia virus (VHSV). The Commission also informed Members that there is no differentiation of genotypes for infection with VHSV and that *C. harengus* consequently should be deleted from the table in Section 2.2.2.

The Commission did not agree to add *Salvelinus fontinalis* and *Lota lota* to Section 2.2.1, because the request was not substantiated by published scientific information.

In response to a comment to change the common name for *Paralichthys olivaceus* to 'Olive flounder', the Commission did not agree. While acknowledging that 'Olive flounder' is a more acceptable name, the Commission wished to remind Members that the Commission uses the common names provided for species in the FAO Terminology Database.

The Commission did not agree to delete the text in Section 2.4.4, Breeding resistant strains, as it contains relevant and useful information.

The Commission did not agree to proposed amendments to Section 3.1, Selection of populations and individual specimens, as the text provides useful guidance. The Section was harmonised with the corresponding Section in Chapter 2.3.4.

Proposed deletions to paragraphs in Section 3.5.1, Samples for pathogen isolation, were not accepted as no rationale was provided and the text was considered valuable.

Technical comments on Section 4.4, Nucleic acid amplification, and Section 4.9.2, Indirect fluorescent antibody test, were referred to the OIE Reference Laboratory experts.

In Section 4.4.1, Real-time RT-PCR, the Commission did not agree with a request to remove mentioning of a commercial kit as the assay was validated using the kit. However, the Commission did note that its preference was to not refer to commercial products unless specific mention is warranted.

The Commission did not agree to add a statement that the test has not been validated to Section 4.7, Real-time RT-PCR, because diagnostic performance has been partially evaluated and performance of the test has been compared with cell culture.

The revised Chapter 2.3.10, Infection with viral haemorrhagic septicaemia virus, is presented in [Annex 14](#) for Member comments. The adoption of the revised chapter has been postponed until the 89th General Session in May 2021. As the revised chapter has already undergone extensive consultation, Members are requested to only submit comments to address substantive issues that have not been considered previously.

EU comment

The EU thanks the OIE and in general supports Annex 14. One comment has been included in section 2.3.2.

8.3. Texts for Member comments

Members were reminded that the Aquatic Animals Commission has commenced the process of progressively reformatting the disease-specific chapters of the *Aquatic Manual* into a new template. As the reformatted and updated chapters have substantial changes, at the last meeting in September 2019, the Commission agreed that only clean versions of the chapters would be provided in the report. Subsequent changes made to these initial revisions following Member comment would be indicated in the usual style (i.e. strikethrough for deletions and double underline for additions).

A software generated document that compares the adopted version of a chapter and the proposed new text will be created. This comparison document will not be included in the Commission's report but will be available on request from the OIE Standards Department (standards.dept@oie.int).

8.3.1. Infection with *Gyrodactylus salaris* (Chapter 2.3.3)

The Commission reviewed Chapter 2.3.3, Infection with *Gyrodactylus salaris*, which had been updated with the assistance of the OIE Reference Laboratory expert and reformatted using the new disease chapter template. The main amendments include an explanatory text on the taxonomy of *G. thymalli* and *G. salaris* and the approach taken in the *Aquatic Manual* to distinguish these species; an updated section on specimen selection, sample collection, transportation and handling; inclusion of an eDNA method for the detection of *G. salaris*; and revised definitions of suspect and confirmed cases in apparently healthy and clinically affected animals.

The revised Chapter 2.3.3, Infection with *Gyrodactylus salaris*, is presented in [Annex 15](#) for Member comments.

EU comment

The EU in general supports the revised Chapter 2.3.3 presented in Annex 15. A couple of EU comments have been included.

8.3.2. Infection with salmonid alphavirus (Chapter 2.3.6)

The Commission reviewed Chapter 2.3.6, Infection with salmonid alphavirus, which had been updated by the OIE Reference Laboratory expert and reformatted using the new disease chapter template. The main amendments include updated Sections on host factors; disease pattern; and specimen selection, sample collection, transportation and handling; updated text on histopathology and cytopathology; and revised definitions of suspect and confirmed cases in apparently healthy and clinically affected animals.

The revised Chapter 2.3.6, Infection with salmonid alphavirus, is presented in [Annex 16](#) for Member comments.

EU comment

The EU supports the proposed changes to Chapter 2.3.6 contained in Annex 16.

8.4. Texts for Member information

8.4.1. Updating the introductory chapters for each host group: amphibians, crustaceans, fish and molluscs (Chapters 2.1.0, 2.2.0, 2.3.0 and 2.4.0, respectively)

The Aquatic Animals Commission identified the need to update the introductory chapters on general information for each host species. Chapter 2.3.0, General information, for fish diseases will be the first chapter to be updated. The Commission will request the assistance of all OIE Reference Laboratory experts for fish diseases in this revision. The experts will be asked to nominate a leader, who would gather input from the group to ensure that the resulting text is as complete and informative as possible. The Commission anticipates circulating a revised Chapter 2.3.0 in its September 2020 report.

8.4.2. The use of environmental DNA methods for aquatic animal disease surveillance

The monitoring of aquatic systems using environmental DNA (eDNA) is a rapidly advancing research field that will provide opportunities for rapid, cost-effective, non-destructive methods to screen for pathogens, especially in wild aquatic populations where samples may be difficult or undesirable to obtain. The Aquatic Animals Commission is aware that eDNA methods exist for detecting pathogenic agents of several listed diseases including *Xenohalotis californiensis*, *Batrachochytrium dendrobatidis*, *Aphanomyces astaci* and *Gyrodactylus salaris*.

The Commission agreed that as these methods are available and currently in use, it would be advisable for guidance to be provided on appropriate application and potential limitations. The Commission noted that as accurate estimates of diagnostic performance are not available for designing surveillance programmes using eDNA assays, data obtained from eDNA methods may not be suitable to support declaration of freedom from listed diseases. The Commission also noted that confirmation of infection by listed diseases could not be made using eDNA methods; however, positive results could be appropriate criteria for a suspect case.

The Commission agreed to develop a guidance document to outline considerations for the appropriate purposes of use, benefits and limitations of eDNA methods. This document is intended to guide appropriate use of eDNA methods and will be reviewed by the Commission at its next meeting in September 2020. The use of an eDNA method for the detection of *Gyrodactylus salaris* is proposed for inclusion in the *Aquatic Manual* chapter for Infection with *Gyrodactylus salaris* (see [Annex 15](#) for Member comment).

9. OIE AD HOC GROUPS

9.1. Electronic *ad hoc* Group on Tilapia lake virus

The Aquatic Animals Commission were informed that the *ad hoc* Group on Tilapia lake virus (TiLV) is continuing its work to evaluate available diagnostic tests for TiLV and they will submit a final report for the Commission's September 2020 meeting.

9.2. *Ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases

The Commission were informed that the *ad hoc* Group on Susceptibility of mollusc species to infection with OIE listed diseases met for the first time in January 2020 to start work on the application of Chapter 1.5, Criteria for listing species as susceptible to infection with a specific pathogen, to OIE listed mollusc diseases. The *ad hoc* Group will meet again electronically in June 2020 to progress its work. The Commission thanked the *ad hoc* Group for its work to date.

10. OIE REFERENCE CENTRES OR CHANGE OF EXPERT

10.1. Evaluation of applications for OIE Reference Centres for Aquatic Animal Health issues or change of experts

The Commission recommended acceptance of the following applications for OIE Reference Laboratory status:

OIE Reference Laboratory for Acute hepatopancreatic necrosis disease

Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, University of Arizona, 1117 E Lowell St., Tucson, Arizona 85721, UNITED STATES OF AMERICA

Tel: +1-520 621 4438

Email: aquapath@cals.arizona.edu ; adhar@email.arizona.edu

Website: www.aquapath.lab.arizona.edu

Designated Reference Expert: Dr Arun Dhar

OIE Reference Laboratory for White spot syndrome virus

Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, University of Arizona, 1117 E Lowell St., Tucson, Arizona 85721, UNITED STATES OF AMERICA

Tel: +1-520 621 4438

Email: aquapath@cals.arizona.edu ; adhar@email.arizona.edu

Website: www.aquapath.lab.arizona.edu

Designated Reference Expert: Dr Arun Dhar

OIE Reference Laboratory for Infectious hypodermal and haematopoietic necrosis

Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, University of Arizona, 1117 E Lowell St., Tucson, Arizona 85721, UNITED STATES OF AMERICA

Tel: +1-520 621 4438

Email: aquapath@cals.arizona.edu ; adhar@email.arizona.edu

Website: www.aquapath.lab.arizona.edu

Designated Reference Expert: Dr Arun Dhar

OIE Reference Laboratory for Hepatobacter penaei (necrotising hepatopancreatitis)

Aquaculture Pathology Laboratory, School of Animal and Comparative Biomedical Sciences, University of Arizona, 1117 E Lowell St., Tucson, Arizona 85721, UNITED STATES OF AMERICA

Tel: +1-520 621 4438

Email: lfarangu@email.arizona.edu

Website: www.aquapath.lab.arizona.edu

Designated Reference Expert: Dr Luis Fernando Aranguren

10.2. Evaluation of annual reports from the OIE Reference Laboratories

Annual reports had been received from all but one OIE Reference Laboratory for diseases of aquatic animals and all Collaborating Centres for aquatic animal issues. A letter would be sent to the laboratory that did not submit a report reminding the expert of his obligations and asking that a report be submitted for review at the September 2020 meeting of the Aquatic Animals Commission.

UNOFFICIAL VERSION

In accordance with the adopted *Procedures for designation of OIE Reference Laboratories* (the SOPs) (<http://www.oie.int/en/scientific-expertise/reference-laboratories/sops/>) and the *Procedures for designation of OIE Collaborating Centres* (<http://www.oie.int/en/scientific-expertise/collaborating-centres/sops/>), the Commission reviewed all the reports received, noting in particular the performance of each Reference Centre with regard to fulfilling the Terms of Reference (ToR) to the benefit of OIE Members.

The Commission noted the significant contributions that had been made by Reference Laboratories during 2019 and wished to thank designated experts for leading these valuable contributions to the OIE mission. The Commission did, however, identify one Reference Laboratory that was not complying with key ToR. The laboratory concerned would be requested to provide an explanation of their situation and possible reasons for the lack of activity; the Delegate will be in copy of all correspondence.

The Aquatic Animals Commission expressed its on-going appreciation for the enthusiastic support and expert advice given to the OIE by the Reference Centres.

10.3. Evaluation of the 5-year work plans from the OIE Collaborating Centres for Disease of Aquatic Animals

The Aquatic Animals Commission reviewed the 5-year work plans that had been received from two of the three Collaborating Centres. The Commission was impressed by the range of activities and their relevance to the OIE Aquatic Animal Health Strategy. The Commission provided feedback on the use of the work plan template to one of these Collaborating Centres. The remaining Centre would be asked to submit a work plan for review at the next Commission meeting in September 2020.

11. OTHER ISSUES

11.1 Improvement of SOP for OIE Register of diagnostic kits

The OIE Secretariat for the Registration of Diagnostic Kits (SRDK) provided a brief overview of its registration procedure and described some recently introduced changes to provide clarity and transparency about the registration procedure itself and to standardise reporting.

The SRDK has also prepared a draft revision to the SOP for OIE Registration of Diagnostic Kits, which describes these changes, and provides expanded background information about the registration requirements. The SOP was presented to the Commission for endorsement.

12. DATE OF NEXT MEETING

The next meeting of the Aquatic Animals Commission is scheduled for 26 August to 2 September 2020.

.../Annexes

**MEETING OF THE OIE
AQUATIC ANIMAL HEALTH STANDARDS COMMISSION
Paris, 19–26 February 2020**

List of participants

MEMBERS OF THE COMMISSION

Dr Ingo Ernst

(President)
Director Aquatic Pest and Health Policy
Animal Division
Department of Agriculture
GPO Box 858 Canberra ACT 2601
AUSTRALIA
Tel.: +61 2 6272 5615
ingo.ernst@awe.gov.au

Dr Kevin William Christison

Department of Agriculture Forestry and Fisheries
Directorate: Aquaculture Research and
Development
Private Bag X 2V
Vlaeberg, 8018
SOUTH AFRICA
KevinCH@daff.gov.za

Dr Alicia Gallardo Lagno

(Vice-President)
Subdirectora nacional de acuicultura
Servicio Nacional de Pesca y Acuicultura
Calle Victoria 2832
CHILE
Tel.: +56 32 281 9282
agallardol@semapesca.cl

Dr Atle Lillehaug

Head of Section
Section for Fish Health and Biosecurity
Norwegian Veterinary Institute
Ullevålsveien 68, 0454 Oslo
Pb 750 Sentrum, N-0106 Oslo
NORWAY
atle.lillehaug@vetinst.no

Dr Prof. Hong Liu

Deputy Director
Animal and Plant Inspection and Quarantine
Technical Center
Shenzhen Customs District
General Administration of Customs,
1011 building of Fuqiang Road
Futianqu, Shenzhen City, Guangdong province
CHINA (People's Rep of)
szc_liuhong@customs.gov.cn
709274714@qq.com

Dr Edmund Peeler

(Vice-President)
Group Manager Aquatic Pest &
Pathogens
CEFAS
Barrack Road, Weymouth
Dorset, DT4 8UB UK
UNITED KINGDOM
Tel.: +44 (0)1305 206746
ed.peeler@cefass.co.uk

OTHE PARTICIPANTS

Dr Chang Siow Foong

Group Director
Professional & Scientific Services
Animal and Veterinary Service
Ministry of National Development
Singapore
chang_siow_foong@nparks.gov.sg

OIE HEADQUARTERS

Dr Gillian Mylrea

Head
Standards Department
g.mylrea@oie.int

Jeannine Fischer

Chargée de mission
Standards Department
j.fischer@oie.int

Dr Stian Johnsen

Chargé de mission
Standards Department
s.johnsen@oie.int

Ms Sara Linnane

Scientific editor
Science Department
s.linnane@oie.int

Dr Gounalan Pavade

Chargé de mission
Science Department
g.pavade@oie.int

CHAPTER 4.X.

**BIOSECURITY
FOR AQUACULTURE ESTABLISHMENTS**

EU comment

The EU thanks the OIE and supports the proposed changes to this chapter.

Article 4.X.1.

Purpose

To provide recommendations on the development and implementation of *biosecurity* measures primarily to mitigate the *risk* of the introduction of specific *pathogenic agents* into *aquaculture establishments*, and if *pathogenic agents* are introduced, to mitigate the *risk* of further spread within, or release from, the *aquaculture establishment*.

Article 4.X.2.

Scope

Biosecurity principles are relevant to application of the standards in the *Aquatic Code* at the level of a country, zone, compartment or *aquaculture establishment* as appropriate. This chapter describes recommendations on *biosecurity* to be applied to *aquaculture establishments*, including semi-open, semi-closed and closed systems. The chapter describes general principles of *biosecurity* planning, categories of *aquaculture* production systems, major transmission pathways, mitigation measures for transmission pathways, the use of the application of risk analysis and approaches for *biosecurity plan* development, to develop a *biosecurity plan*, and the key components of a plan.

For further guidance on disease prevention and control refer to Section 4 of the *Aquatic Code*.

Article 4.X.3.

Introduction

~~The fundamental measures that underpin aquatic animal disease prevention at the level of country, zone or compartment is the application of *biosecurity*. *Biosecurity* at the level of an *aquaculture establishment* is integral to effective *biosecurity* at the level of a country, zone or compartment and thus the optimal health status and welfare of aquatic animal populations.~~ This chapter describes *biosecurity* principles designed to mitigate the risks associated with the introduction of *pathogenic agents* into, the spread within, or the release from *aquaculture establishments*. ~~The application of *biosecurity* at the level of an *aquaculture establishment* may be integral to effective *biosecurity* at the level of a country, zone or compartment to maintain the optimal health status of *aquatic animal* populations.~~

Given the unique challenges posed by varied *aquaculture* production systems and the vast diversity of farmed *aquatic animal* species, the development of *biosecurity plans* for *aquaculture establishments* requires the assessment of *disease risks* posed by specific *pathogenic agents* and their potential transmission pathways. A *biosecurity plan* describes physical and management measures to mitigate the identified risks according to the circumstances of the *aquaculture establishment*. *Aquaculture establishment* staff, and service providers and *aquatic animal health professionals or veterinarians* should be engaged in developing and implementing the *biosecurity plan* to ensure it is practical and effective.

The outcome achieved through the implementation of *biosecurity* at *aquaculture establishments* is improved health and welfare status of *aquatic animals* throughout the production cycle. The benefits may include improved market access and increased productivity, directly through improved survival, growth rates and feed conversion and indirectly

through the a reduction in the use in treatments of veterinary medicinal products (including *antimicrobial agents*) and associated production costs.

Article 4.X.4.

General principles

Biosecurity is a set of physical and management measures which, when used together, cumulatively reduce the *risk of infection* in *aquatic animal* populations at within an *aquaculture establishment*. Planning and implementation of biosecurity within an aquaculture establishment requires planning to identify risks and consider cost effective cost-effective measures to achieve the identified biosecurity objectives of the plan. The measures required will vary among between aquaculture establishments, depending on factors such as risk likelihood of exposure to pathogenic agents, the species of aquatic animal farmed species, the category of aquaculture production system, husbandry practices and geographical location. Although different Different approaches may be used to achieve an identified biosecurity objective, however, the general principles for developing and implementing a biosecurity plan are consistent and are described as below:

- 1) Planning is necessary to document the objectives of the biosecurity plan, the identified risks to be managed, the measures that will be put in place to manage the disease risks, required operating procedures and monitoring, as described in Articles 4.X.6. and 4.X.7.
- 2) Potential pathways for pathogenic agents to be transmitted into, spread within and released from the aquaculture establishment must be identified, as described in Articles 4.X.5. and 4.X.6., and giving consideration to the category of aquaculture production system and design of the aquaculture establishment.
- 3) Risk analysis should be undertaken to identify and evaluate biosecurity disease threats and ensure that the plan addresses risks appropriately and efficiently. The risk analysis may range from a simple to a complex analysis depending on the objectives of the biosecurity plan and, the circumstances of the aquaculture establishment and the disease risks, as described in Article 4.X.7.
- 4) Biosecurity measures to address identified disease risks should be evaluated based on the basis of their potential effectiveness, initial and ongoing costs (e.g. building works, maintenance), and management requirements, as described in Article 4.X.7.
- 4) Management practices should be integrated into the aquaculture establishment's operating procedures and associated relevant training are is provided to personnel, as described in Articles 4.X.7. and 4.X.8.
- 5) Clear signage should be displayed to promote awareness and compliance with biosecurity plan measures by personnel, visitors and the public.
- 6) Appropriate records and documentation are essential to demonstrate effective implementation of the biosecurity plan. Examples are provided in Article 4.X.8.
- 7) A routine review schedule for routine reviews and audits of the biosecurity plan should be described. and identified triggers for ad-hoc ad hoc review must be determined (e.g. outbreaks of disease, and changes to infrastructure, production techniques, disease outbreaks, or risk profiles). Third party audits may be required where recognition of the biosecurity measures is required by customers, or regulators, or for market access, as described provided in Article 4.X.8.

Article 4.X.5.

Categories of aquaculture production systems

Aquatic animals can be produced in Four different categories of aquaculture production systems, which are defined based on the capacity to treat water entering and exiting the system, and the level of control of over aquatic animals and vectors. These measures factors need to be considered in *biosecurity* planning.

Open systems

In an open Open aquaculture production systems, it is not possible to have no control of the water, environmental conditions, and animals and or vectors. These production systems may include stock enhancement of wild populations with aquatic animals originating from aquaculture establishments or from the wild. As these systems cannot be considered 'aquaculture establishments', they are not considered further in this chapter. However, movements of aquatic animals from aquaculture establishments to open systems should still be subject to assessed to determine the need for disease mitigation measures.

Semi-open systems

In a semi-open aquaculture production system, it is not possible to have control over the water entering or exiting the system, or of over the environmental conditions. Some aquatic animals and vectors may also enter and exit the system. Examples of semi-open aquaculture production systems are net pens for finfish and suspended baskets for molluscs aquaculture in natural water bodies and mollusc aquaculture, either suspended in the water column or on the ocean floor.

Semi-closed systems

In a semi-closed aquaculture production system, there is some control of over the water entering and exiting the system and of over the environmental conditions. Aquatic animals and vectors may be prevented from entering and exiting the system; however, there is limited control to prevent the entry or exit of pathogenic agents. Examples of semi-closed aquaculture production systems are ponds, raceways, enclosed floating pens, and flow-through tanks.

Closed systems

In a closed aquaculture production system, the there is sufficient control of over water entering and exiting the system can to exclude aquatic animals, vectors and pathogenic agents. Environmental conditions can also be controlled. Examples of closed aquaculture systems include recirculating aquaculture production systems, production systems with a safe water supply free from pathogenic agents or aquatic animals (e.g. ground water), or those with high levels of treatment (and redundancy) of water entering or exiting the system.

Article 4.X.5. bis

Area management

It may not be possible to control the transmission of pathogenic agents among semi-open or semi-closed aquaculture establishments that are in close proximity within shared water bodies. In these circumstances, a consistent set of biosecurity measures should be applied by all of the aquaculture establishments considered to be epidemiologically linked. Area management agreements can formalise the coordination of common biosecurity measures among all of the epidemiologically linked aquaculture establishments.

Article 4.X.6.

Transmission pathways and associated risks and mitigation measures

Pathogenic agents can move into, spread within, and be released from aquaculture establishments via various transmission pathways. The identification of all potential transmission pathways is essential for the development of an effective biosecurity plan. Mitigation of pathways that are likely to result in transmission of specific may expose susceptible aquatic animals to high loads of pathogenic agents should be prioritised for mitigation.

The risks associated with the introduction into, spread within, and release of pathogenic agents from the aquaculture establishment need to be considered for each of the following transmission pathways.

Annex 2 (contd)

1. Aquatic animals

Movement of *aquatic animals* into, within and from *aquaculture establishments*, either intentionally or unintentionally, ~~may usually may pose~~ ~~pose has~~ a high likelihood risk of *pathogenic agent* transmission. This is particularly the case when clinically and sub-clinically infected *aquatic animals*, or *aquatic animals* with unknown health status are moved into a susceptible population.

~~Aquatic animals intentionally brought introduced into, or moved within, an aquaculture establishment, or moved within it,~~ may include broodstock, juvenile stock for on-growing, and genetic material such as eggs. Both horizontal and vertical transmission mechanisms of pathogenic agents should be considered for *aquatic animals*. ~~The risk of transmitting pathogenic agents via aquatic animals should be managed; possible mitigation measures include the giving consideration to the following mitigation measures can be managed by:~~

- a) Only introducing introduce into the *aquaculture establishment* *aquatic animals* with a known health status ~~into the aquaculture establishment with known health status~~, which is of equal or higher status than the existing animals in the establishment.
- b) ~~Quarantining~~ Placing introduced ~~if~~ *aquatic animals* of unknown *disease* status are introduced, they should be placed into quarantine from other farm populations in separate production units or dedicated quarantine facilities.
- c) Where appropriate, treating treat ~~treatment of~~ *quarantined aquatic animals* to mitigate *disease risks* (for example, treatment for external parasites).
- d) Ensuring Ensure biosecure transport of *aquatic animals* that avoids exposure to *pathogenic agents*.
- e) Only moving move *aquatic animals* between different populations within the establishment following consideration of the *disease risks* and with a view to maintaining the highest possible health status of the *aquatic animal* population.
- f) Isolating Isolate *aquatic animal* populations that display clinical signs of *disease* from other populations until the cause is known and the situation is resolved.
- g) Removing Remove sick or dead *aquatic animals* from production units as soon as possible and disposing dispose of them in a biosecure manner in accordance with Chapter 4.7.
- h) Reporting of Report unexplained or unusual mortalities, or suspicion of a notifiable *disease* in *aquatic animals* to the *Competent Authority* in accordance with local requirements. Investigation and *diagnosis* of the cause of mortality should be undertaken.
- i) If possible, totally depopulating depopulate the *aquaculture establishment* at intervals, for instance between *aquatic animal* generations or production cycles, followed by cleaning and *disinfection* of production installations. Sites should be fallowed for a period sufficient to interrupt *infection* cycles and reduce or eliminate pathogen challenge to restocked *aquatic animals*. Fallowing should be coordinated for *aquaculture establishments* that are epidemiologically linked through shared water bodies.
- j) Where possible, preventing unintended movement of aquatic animals into, within or from the establishment. Considering Consider physical measures to minimise the likelihood of escape of farmed *aquatic animals* or the entry of wild *aquatic animals* into the *aquaculture establishment*. The likelihood of entry or escape of *aquatic animals* will be higher for semi-open than for closed or semi-closed systems.

The *risk* of unintentional movements of *aquatic animals* will be influenced by the category of *aquaculture* production system, with the likelihood being higher for semi-open than closed systems. If *risks* are found to be high, physical mitigation measures may be necessary.

2. Aquatic animal products and aquatic animal waste

Aquatic animal products may also be brought into, ~~moved within and~~ or moved out of an *aquaculture establishments* or moved within it; for example, *aquatic animal products* derived from *aquatic animals* harvested at other sites. *Aquatic animal waste* ~~waste~~ may include the ~~be generated~~ ~~entire body or parts of~~ ~~when~~ *aquatic animals* that have died or been killed for disease control purposes, as or ~~when they~~ ~~through killing and~~ ~~processing of aquatic animals~~ ~~have been killed and processed~~ and their parts, that are not intended for human consumption or ~~other purposes~~.

Movement of *aquatic animal products* and *aquatic animal waste* ~~waste~~ into, within ~~and out of~~ or from *aquaculture establishments* may pose a risk of *pathogenic agent* transmission. This is particularly the case when a susceptible population is exposed to *aquatic animal products* and *aquatic animal waste* ~~waste~~ derived from clinically or sub-clinically infected *aquatic animals*. ~~High risk waste includes aquatic animal waste waste that constitutes, or is suspected of constituting, a high significant health risk to aquatic animals.~~ Movement of *aquatic animal waste* into *aquaculture establishments* should be avoided where possible. Waste should be stored, transported, disposed of and treated following the guidance in Chapter 4.7. Handling, disposal and treatment of *aquatic animal waste*.

For intentional movements of *aquatic animal products* and *aquatic animal waste* ~~waste~~, the likelihood of presence of *pathogenic agents* in the *aquatic animals* from which products *aquatic animal products* and waste are derived should be evaluated giving consideration to the species, source, and health status.

The risk of transmitting *pathogenic agents* via *aquatic animal products* and *aquatic animal waste* ~~waste~~ should be ~~assessed and~~ managed; ~~possible mitigation measures include the~~ giving consideration to the following ~~mitigation measures~~ can be managed by:

- a) ~~determining~~ **Determine** the potential *disease risk* of *aquatic animal products* and *aquatic animal waste* to *aquatic animals* in the establishment and the environment;
- b) **Manage** *aquatic animal products* and *aquatic animal waste* in areas within the *aquaculture establishment* that are ~~isolated~~ ~~isolating~~ ~~areas within the aquaculture establishment where aquatic animal products and aquatic animal waste waste are managed~~ from *aquatic animal* populations to minimise identified *disease transmission risks*;
- c) **ensure** ~~procedures~~ ~~systems~~ are implemented for appropriate collection, treatment (inactivating *pathogenic agents*), transport, storage or disposal of *aquatic animal products* and *aquatic animal waste* ~~waste~~ to minimise ~~identified disease transmission risks~~ ~~the risks of transmitting pathogenic agents~~.

3. Water

Water is an important asset that supports productivity and *aquatic animal* health but may present a risk of ~~the~~ introduction of *pathogenic agents* into, spread within, and release from *aquaculture establishments*. The source of the water, and how it ~~may~~ provides an epidemiological link between the *aquaculture establishment* and other farmed or wild populations or processing plants, should be identified and considered. Exposure to transport water and ballast water should be considered.

The risk of the *aquaculture establishment* being exposed to water containing *pathogenic agents* may be influenced by the category of *aquaculture* production system, the likelihood being higher for semi-open than for closed systems. Any water that is flowing from *aquatic animals* with lower or unknown health status presents a potential risk of transmitting *pathogenic agents* to *aquatic animals* of a higher health status.

The risk of transmitting *pathogenic agents* via water should be ~~assessed, and~~ managed; ~~possible mitigation measures include the~~ giving consideration to the following ~~mitigation measures~~ can be managed by:

Annex 2 (contd)

- a) Where possible, choosing choose water sources that are entirely free of susceptible *aquatic animal* populations and *pathogenic agents* of concern. Such water sources may include saline or fresh groundwater, de-chlorinated municipal water, and artificial seawater. These water sources may be particularly suitable for high health status *aquatic animals* such as broodstock.
- b) Providing Provide an appropriate level of screening, filtration or *disinfection* (in accordance with Chapter 4.3.) of water from sources that are likely to contain *susceptible species* and which may present a *risk* of *pathogenic agent* transmission (e.g. oceans, streams or lakes). The type and level of treatment required will depend on the identified *risks*.
- c) Provide an appropriate level of filtration and disinfection or holding (in accordance with Chapter 4.3.) of effluent water (and associated filtered waste) from aquaculture establishments (or associated slaughterhouses or processing facilities) where it may present a risk of pathogenic agent transmission to wild aquatic animals or other aquaculture establishments with susceptible species. The type and level of treatment required will depend on the identified risks.
- ed) Ensuring Ensure the position of water intakes and outlets for semi-closed and closed *aquaculture establishments*, and the location of semi-open *aquaculture establishments*, minimises contamination from other farmed or wild populations or processing plants, taking into account factors such as distance and water currents.
- e) The likelihood of ingress of contaminated water either through flooding from external sources or from defective infrastructure (e.g. leaking pipes, blocked drains, bund wall failure) should be assessed and appropriate management or infrastructure measures applied.
- f) Assess the risk and establish procedures to treat and dispose of waste water resulting from the transport of aquatic animals.

4. Feed

Feed can be an important pathway for transmission of *pathogenic agents* to *aquatic animals*. *Feed* may be initially infected with *pathogenic agents* or contaminated during harvest, transport, storage and processing of *commodities* used as *feed ingredients*. Poor hygiene may contribute to contamination during manufacture, transport, storage and use of *feed*.

In closed or semi-closed production systems there can be a high level of control on *aquatic animal feeds*. However, in semi-open production systems, *aquatic animals* may obtain food from their environment (e.g. filter-feeding molluscs or predation of wild fish which may be preyed on predated by farmed fish in net pens).

The *risk* of transmitting *pathogenic agents* via *aquatic animal feed* ~~can~~ should be assessed, and managed by mitigation measures as ~~described~~ provided in Chapter 4.8., for example using *feed* and *feed ingredients* that:

- a) have undergone sufficient processing to inactivate *pathogenic agents* of concern;
- b) are from sources that are declared free from the *pathogenic agents* of concern or have been confirmed (e.g. by testing) that *pathogenic agents* are not present in the feed or feed ingredients commodity;
- c) have been processed, manufactured, stored, ~~and~~ transported and delivered during feeding to aquatic animals in a manner to prevent contamination by *pathogenic agents*.

5. Fomites

Equipment, *vehicles*, packaging material, clothing, footwear, sediments, infrastructure and other fomites can mechanically transfer *pathogenic agents* into, within and from an *aquaculture establishment*.

The level of risk likelihood of transferring *pathogenic agents* will depend on the stability of the pathogenic agent in the environment, the presence and nature of organic matter on the fomite surface, as well as the type of surface and its ability capacity to hold water. The risk likelihood of transferring *pathogenic agents* may be higher for fomites which are difficult to clean and disinfect. Sharing eEquipment that is shared between *aquaculture establishments*, between *aquaculture establishments* and processing facilities, or between different production units with of unequal health status within an *aquaculture establishment* with unequal health status, may result in the spread of pathogenic agents present a higher risk than compared to new or dedicated equipment. The risk risk likelihood of transmitting *pathogenic agents* via fomites should be assessed and managed; possible mitigation measures include the giving consideration to the following mitigation measures can be managed by:

- a) Assessing Assess the disease risk associated with any fomites brought moved into, within or from the *aquaculture establishment* for their disease risk.
- b) Ensuring Ensure procedures and infrastructure are in place to clean and disinfect fomites, including at designated delivery and loading areas, prior to entry into the aquaculture establishment. Recommendations for the cleaning and disinfection disinfection of fomites are described in Chapter 4.3.
- c) Assigning dedicated equipment for use in production units of different health status. Where equipment must be used in multiple production units it should be cleaned and disinfected prior to movement between units.
- c) Wherever possible, dedicating Dedicate, where possible, items that are difficult to disinfect, or those with a high likelihood of contamination, to a specific *aquaculture establishment* rather than instead of moving them between *aquaculture establishments* after disinfection.
- d) Applying Apply the mitigation measures described at points a) to c) above to the movement of fomites between production units within an aquaculture establishment with the measures determined based on an evaluation of the risk of disease transmission disease risks.

6. Vectors

Vectors can transport *pathogenic agents* to susceptible *aquatic animals* in *aquaculture establishments*. These They include wild *aquatic animals* entering via the water supply, predators, wild birds, and scavengers, pest animals such as rodents, and people. *Vectors* can also transfer *pathogenic agents* into, within and from an *aquaculture establishment*, either by mechanical transfer or as a developmental stage of the pathogenic agent within the vector. The risk of unintentional exposure to vectors will be influenced by the category of aquaculture production system.

The risk likelihood of transferring *pathogenic agents* via *vectors* varies with the type of vector species, the nature of the *pathogenic agent*, the category of *aquaculture* production system, and the level of *biosecurity*. Measures identified to mitigate risks associated with aquatic animals, as described in point 1, can also be applied to mitigate risks associated with vectors. Mitigation measures for other vectors include:

The risk of transmitting pathogenic agents via vectors should be assessed, and managed giving consideration to the following mitigation measures:

- a) netting (to prevent access by birds): Physical mitigation measures should be used to prevent the access of vectors to aquaculture establishments including may include:

Annex 2 (contd)

- i) filtering or screening of water entering and exiting semi-closed and closed aquaculture production systems to prevent entry of wild aquatic animals;
- ii) surrounding land-based aquaculture production systems by a fence or a wall to prevent entry of animals and people, with a gate for controlled access;
- iii) surrounding floating aquaculture production systems by barriers on the establishment perimeter to prevent contact with or entry of wild aquatic animals and other animals;
- iv) covering outdoor aquaculture production systems with nets to prevent access by birds.
- b) barriers on the establishment perimeter to prevent entry by of other animals (e.g. electric fencing);
- b) Controlling Access of personnel to aquaculture establishments should be controlled by creating a defined border between the outer risk area and the inner biosecure area comprising facilities for:
 - i) changing of clothes and shoes, or use of disposal coverings (hoods, coats, shoe coverings);
 - ii) disinfection of hands, and the use of foot baths for shoe disinfection.
- eb) Ppest control. and secure storage of feed and mortalities.

7. Personnel and visitors

- a) Access of personnel and visitors to aquaculture establishments should be controlled by creating a defined border between the outer risk area and the inner biosecure area comprising facilities for:
 - i) changing of clothes and shoes, or use of disposable coverings (hoods, coats, shoe coverings);
 - ii) disinfection of hands, and the use of foot baths for shoe disinfection.
- b) All visitors should be briefed and supervised to ensure compliance with the biosecurity plan.

Article 4.X.7.

Risk analysis

Risk analysis is an accepted approach for evaluating *biosecurity* threats and is used to support the development of mitigation measures. A formal *risk analysis* has four components: *hazard* identification, *risk assessment*, *risk management* and *risk communication* (see Chapter 2.4.). This article elaborates the principles in Chapter 2.1. and applies them for the development of biosecurity plans for aquaculture establishments.

A *biosecurity plan* may not necessarily require a comprehensive *risk analysis* to evaluate *disease risks* linked to transmission pathways. The chosen approach may depend on the objectives of the *biosecurity plan*, the level of *biosecurity* that is appropriate for the specific production requirements of the *aquaculture establishment*, the complexity of the threats to be addressed, and the availability of information and resources. Depending on these circumstances, a partial analysis may be appropriate, and can build on previous experiences to identify the *hazards* associated with relevant transmission pathways.

The three formal steps of the *risk analysis* process to underpin a *biosecurity plan* are:

Step 1 – Hazard identification

Hazard identification determines which *pathogenic agents* should be the subject of the *risk assessment*. A hazard may include a specific pathogenic agent or be defined in more general terms as a group of pathogenic agents. This step includes identifying and collecting relevant information on the *pathogenic agents* that have a potential to cause *diseases* in *aquatic animal* populations within an *aquaculture establishment*. This process must consider the *aquatic animal health status* of the establishment and, for semi-open and semi-closed *aquaculture* production systems, the *aquatic animal health status* of the epidemiologically linked environments. The following step is to identify both known and emerging diseases, not present in the aquaculture establishment, which may negatively impact the farmed population. Known and emerging diseases which could negatively impact the farmed population should be identified, regardless of whether they are present in the aquaculture establishment.

To complete the next steps of the *risk assessment*, ~~required~~ information on the identified *hazards* is ~~required~~ needed and includes: i) the frequency of occurrence, ii) the biophysical characteristics, iii) the likelihood of detection if present and iv) the possible transmission pathways (described in Article 4.X.6.). Many of the hazards will share the same pathways. A *hazard* may include a specific *pathogenic agent* or be defined in more general terms as a group of *pathogenic agents*.

Step 2 – Risk Assessment

A *risk assessment* can be initiated once it has been identified that a *biological hazard* exists, and the required information listed under step 1 has been gathered. The aim of the *risk assessment* is to establish a *risk* estimate, which is the product of the likelihood and consequences of entry of a pathogenic agent ~~entry~~ into, spread within or release from the *aquaculture establishment*.

A *risk assessment* can be quantitative or qualitative. Both methods require the same conceptual pathway which identifies the necessary steps for *hazard* introduction, establishment and spread to be constructed. In a qualitative assessment, introduction and establishment are estimated using descriptors of likelihood. A quantitative assessment requires data on which to estimate likelihood. In most circumstances, the likelihood of disease transmission and associated consequences pathways will be assessed qualitatively but within a formal *risk assessment* framework. Examples of descriptors for qualitative estimates of likelihood and consequence are given in Tables 1 and 2. Table 3 illustrates how estimates of likelihood and consequence can be combined in a matrix to give an estimate of *risk*.

Table 1. Qualitative descriptors of likelihood

Estimate	Descriptor
Remote	<u>Never heard of</u> <u>Very unlikely</u> , but not impossible.
Unlikely	May occur here, but only in rare circumstances.
Possible	Clear evidence to suggest this is possible in this situation.
Likely	It is likely, but not certain, to occur here.
Certain	It is certain to occur.

Table 2. Qualitative descriptors of consequences

Estimate	Descriptor <u>of consequences at level of the aquaculture establishment</u>
Insignificant	Impact not detectable or minimal. <u>No trade impact.</u>
Minor	Impact <u>Limited decreased production on aquaculture establishment productivity limited to some affecting only a small number of production units or short-term, and/or very limited and transitory disruption to trade, only.</u>
Moderate	Widespread impact on aquaculture establishment productivity due to increased mortality or decreased performance. <u>Decreased production (e.g. sustained increased mortality or decreased growth rate) and/or some short-term to medium-term disruption to trade, resulting in financial loss.</u>
Major	Considerable, decreased impact on aquaculture establishment production, and/or some medium-term to long-term disruption to trade, resulting in significant financial loss, resulting in serious supply constraints and financial impact.
Catastrophic	Complete depopulation production loss, in of the aquaculture establishment and possibly barriers to resumption of production, and/or complete loss of trade, resulting in extreme financial loss.

Annex 2 (contd)

Table 3. Matrix for assessing estimating risk

Likelihood estimate	Consequence rating				
	insignificant	minor	moderate	major	catastrophic
remote	negligible	low	low	low	medium
unlikely	low	low	medium	medium	high
possible	low	medium	medium	high	high
likely	low	medium	high	high	extreme
certain	medium low	high	high	extreme	extreme

Results of risk assessments informs which biological hazards need to be addressed, which critical control points on the transmission pathway should be targeted, and the measures which that are most likely to be effective in reducing risk.

Table 4. Interpretation of risk estimates

<u>Risk level estimate</u> *	Explanation and management response
Negligible	Acceptable level of <i>risk</i> . No action required.
Low	Acceptable level of <i>risk</i> . On-going monitoring may be required.
Medium	Unacceptable level of <i>risk</i> . <u>Active management</u> <u>Review and strengthen the risk mitigation measures is required to reduce the level of risk.</u>
High	Unacceptable level of <i>risk</i> . <u>Intervention</u> <u>Identify and implement additional risk mitigation measures is required to mitigate the risk.</u>
Extreme	Unacceptable level of <i>risk</i> . <u>Take immediate action to mitigate the risk.</u> <u>Urgent intervention is required to mitigate the level of risk.</u>

*The Risk level estimate is determined by from a combination of the likelihood and consequence estimates score obtained using the risk matrix (Table 3). Likelihood and consequence estimates are combined using the risk matrix (Table 3) to produce the risk estimate.

Step 3 – Risk Management

Risk management is used to determine the appropriate management response for the assessed level of *risk* as described in Table 4. The *risk assessment* process identifies the steps within transmission pathways necessary for a *risk* to be realised and thus allows the most effective mitigation measures to be determined. Many of the *hazards* will share the same pathways and thus therefore mitigation measures may be effective against more than one *hazard*. Information on hazards and their pathways of introduction (step 1) should be combined with an assessment of risk associated with each the assessment of the pathways (step 2) to identify the most appropriate and cost-effective risk mitigation measures.

Article X.X.6. describes some possible mitigation measures relevant for to different transmission pathways. The most appropriate mitigation measures for a specific *aquaculture establishment* will depend on the risks hazards identified, the effectiveness and reliability of the mitigation measure, the category of *aquaculture* production system and cost.

After the implementation of the *biosecurity plan*, *hazards* should be regularly reassessed, and measures adjusted according to any changed *risk* estimates.

Article 4.X.8.

Biosecurity plan development

The purpose of a *biosecurity plan* is primarily to reduce the *risk* of introducing *pathogenic agents* into an *aquaculture establishment*, and if *pathogenic agents* are introduced, to reduce the *risk* of further spread within or release from the *aquaculture establishment*. The plan will document identified transmission pathways and the outputs of any *risk analysis* performed (*hazards*, *risk estimate* and mitigation measures), and information relevant to ongoing implementation, monitoring and review of the plan.

1. Development of a biosecurity plan

The process of developing a *biosecurity plan* will vary depending on its objectives of the *biosecurity plan*, the level of *biosecurity* appropriate to the specific production system requirements, the complexity of the *disease risks* to be addressed, and availability of information and resources. Consideration and documentation of the following issues are recommended:

- a) objectives, scope and regulatory requirements for the *biosecurity plan*;
- b) information about the *aquaculture establishment* including an up-to-date plan of the layout of buildings and production units (including epidemiological units, if any, and structures and the processes to maintain separation methods), loading/unloading, unpacking, processing, feed storage, waste aquatic animal waste storage, reception areas, access points and maps showing major movements of *aquatic animals*, *aquatic animal products* and *aquatic animal waste*, water, feed and fomites (including staff, equipment and vehicles);
- c) the potential pathways for entry of *pathogenic agents* into, spread within or release from the *aquaculture establishment* (refer to Article X.X.6. above);
- d) a *risk analysis*, including identification of the major *disease hazards* to the *aquaculture establishment* (refer to Article X.X.7. above);
- e) the mitigation measures that have been determined to address identified *risks*;
- f) emergency procedures in the event of a *biosecurity* failure. These may include reporting requirements, and emergency measures to eradicate pathogenic agents such as aquatic animal depopulation and disposal, and site disinfection, in accordance with Chapters 4.3. and 7.4.;
- g) ~~standard operating procedures required to support implementation of the mitigation measures, emergency procedures and the training requirements of personnel;~~
- gh) internal and external communication procedures, and roles and responsibilities of personnel, aquaculture establishment staff and essential contact information, e.g. for personnel, staff, farm veterinarian and the Competent Authority;
- hi) monitoring and audit schedule;
- hj) performance evaluation;
- ij) standard operating procedures required to support all implementation of the mitigation measures described by the biosecurity plan, emergency procedures and the training requirements of establishment personnel.

Annex 2 (contd)2. Key components of a biosecurity plan

a) Standard operating procedures (SOPs)

SOPs describe routine management processes that must be performed to support the effectiveness of the *biosecurity plan*. Each SOP should clearly describe its objectives, staff responsibilities, the procedure (including record keeping), precautions and a review date.

Staff should be trained in the application of the SOPs including completion of forms, checklists and other records associated with each procedure, as well as routine communication requirements.

b) Documentation and record keeping

The *biosecurity plan* describes the documentation necessary to provide evidence of compliance with the mitigation measures plan. The level of detail required in the documentation depends on the outcomes of the transmission pathway assessment.

Examples of documentation required may include: *aquaculture establishment* layout, movements of *aquatic animals*, escapees, origin and destination and health status of the *aquatic animals* introduced to the *aquaculture establishment*, quarantine measures, records of visitors to the establishment, escapees, stocking densities, feeding and growth rates, records of staff training, treatments/vaccination, water quality, cleaning and disinfection events, morbidity and mortality (including removal and disposal of mortalities), surveillance and laboratory records.

c) Emergency procedures

Procedures should be developed and, when necessary, implemented to minimise the impact of emergencies, *disease* events, or unexplained mortality in *aquatic animals*. These procedures should include clearly defined thresholds that help to identify an emergency incident and activate response protocols, including reporting requirements.

d) Health monitoring

Health monitoring as part of the *biosecurity plan* involves monitoring of the health status of *aquatic animals* in *aquaculture establishments*. Activities may include *disease surveillance*, routine monitoring of stock for important health and production parameters (e.g. by staff, an *aquatic animal health professional* or a veterinarian), recording of clinical signs of *disease*, morbidity and mortality, and analysis of these data (e.g. calculation of rates of morbidity and mortality and diseases).

e) Routine review and auditing

The *biosecurity plan* should describe a systematic auditing schedule to verify implementation and compliance with the requirements of the *biosecurity plan*. Routine revision of the *biosecurity plan* is necessary to ensure that it continues to effectively address *biosecurity risks*.

The *biosecurity plan* should also be reviewed at least annually or in response to changes to the *aquaculture establishment* operations, changes in facility design, changes ~~to~~ in husbandry approaches, identification of a new *disease risk*, or the occurrence of a *biosecurity* incident. *Biosecurity* incidents, and actions taken to remedy them, should be documented to enable SOP re-assessments of SOPs.

CHAPTER 1.3.

DISEASES LISTED BY THE OIE

EU comment

The EU thanks the OIE and supports the proposed changes to this chapter.

[...]

Article 1.3.3.

The following *diseases* of crustaceans are listed by the OIE:

- Acute hepatopancreatic necrosis disease
 - Infection with *Aphanomyces astaci* (crayfish plague)
 - Infection with *Hepatobacter penaei* (necrotising hepatopancreatitis)
 - Infection with infectious hypodermal and haematopoietic necrosis virus
 - Infection with infectious myonecrosis virus
 - Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)
 - ~~Infection with shrimp haemocyte iridescent virus~~ Infection with decapod iridescent virus¹
 - Infection with Taura syndrome virus
 - Infection with white spot syndrome virus
 - Infection with yellow head virus genotype 1.
-

**Model Article 10.X.13. for the fish disease-specific
Chapters 10.5,
10.6. and 10.10. (and Article 10.4.17. for
Chapter 10.4. Infection with infectious
salmon anaemia virus)**

EU comment

The EU thanks the OIE and supports the proposed changes to this chapter.

[...]

Article 10.X.13.

Importation of disinfected eggs for aquaculture from a country, zone or compartment not declared free from infection with [pathogenic agent X]

- 1) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, zone or compartment not declared free from infection with [pathogenic agent X], the *Competent Authority* of the *importing country* should assess in accordance with Chapter 4.4 the risk associated with at least the following:
 - a) the ~~infection with pathogenic agent X~~ likelihood that status of the water to be used during the *disinfection* of the eggs is contaminated with [pathogenic agent X];
 - b) the prevalence of infection with [pathogenic agent X] in broodstock (including by results from testing of ovarian fluid and milt); and
 - c) the temperature and pH of the water to be used for *disinfection*.
- 2) If the *Competent Authority* of the *importing country* concludes that the importation is acceptable, it should request that apply the following risk mitigation measures are applied, including:
 - a) disinfection of the eggs prior to importing, in accordance with recommendations in Chapter 4.4. ~~of those specified by the Competent Authority of the importing country~~;
 - b) between *disinfection* and importation ~~the import~~, eggs should not come into contact with anything which may affect their health status.

The *Competent Authority* should consider internal measures, such as additional ~~renewed~~ *disinfection* of the eggs upon arrival in the *importing country*.
- 3) When importing disinfected eggs of the species referred to in Article 10.X.2. for *aquaculture*, from a country, zone or compartment not declared free from infection with [pathogenic agent X], the *Competent Authority* of the *importing country* should require that the consignment be accompanied by an *international aquatic animal health certificate* issued by the *Competent Authority* of the *exporting country* certifying that the procedures described in point 2 a) and b) of this article have been fulfilled.

[...]

CHAPTER 10.9.

INFECTION WITH SPRING VIRAEMIA OF
CARP VIRUS**EU comment**

The EU thanks the OIE and supports the proposed changes to this chapter.

[...]

Article 10.9.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.:

~~all varieties and subspecies of common carp (*Cyprinus carpio*), bighead carp (*Aristichthys nobilis*), bream (*Abramis brama*), Caspian white fish (*Rutilus kutum*), fathead minnow (*Pimephales promelas*), golden shiner (*Notemigonus crysoleucas*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*), roach (*Rutilus rutilus*) and sheatfish (also known as European or wels catfish) (*Silurus glanis*).~~

<u>Family</u>	<u>Scientific name</u>	<u>Common name</u>
Cyprinidae	<i>Abramis brama</i>	Bream
	<i>Aristichthys nobilis</i>	Bighead carp
	<i>Carassius auratus</i>	Goldfish
	<i>Ctenopharyngodon idella</i>	Grass carp
	<i>Cyprinus carpio</i>	Common carp (all varieties and subspecies)
	<i>Danio rerio</i>	<u>Zebrafish</u>
	<i>Notemigonus crysoleucas</i>	Golden shiner
	<i>Pimephales promelas</i>	Fathead minnow
	<i>Rutilus kutum</i>	Caspian white fish
	<i>Rutilus rutilus</i>	Roach
Siluridae	<i>Silurus glanis</i>	Sheatfish (also known as European or wels catfish) <u>Wels catfish</u>

[...]

CHAPTER 10.10.

INFECTION WITH VIRAL HAEMORRHAGIC
SEPTICAEMIA VIRUS**EU comment**

The EU thanks the OIE and supports the proposed changes to this chapter.

[...]

Article 10.10.2.

Scope

The recommendations in this chapter apply to the following species that meet the criteria for listing as susceptible in accordance with Chapter 1.5.: rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*), grayling (*Thymallus thymallus*), white fish (*Coregonus* spp.), pike (*Esox lucius*), turbot (*Scophthalmus maximus*), herring and sprat (*Clupea* spp.), Pacific salmon (*Oncorhynchus* spp.), Atlantic cod (*Gadus morhua*), Pacific cod (*Gadus macrocephalus*), haddock (*Gadus aeglefinus*) and rockling (*Onos mustelus*). These recommendations also apply to any other susceptible species referred to in the Aquatic Manual when traded internationally.

<u>Family</u>	<u>Scientific name</u>	<u>Common name</u>	<u>Genotype</u>
<u>Ammodytidae</u>	<u><i>Ammodytes hexapterus</i></u>	<u>Pacific sand lance</u>	<u>IVa</u>
<u>Aralichthyidae</u>	<u><i>Paralichthys olivaceus</i></u>	<u>Bastard halibut</u>	<u>IVa</u>
<u>Carangidae</u>	<u><i>Trachurus mediterraneus</i></u>	<u>Mediterranean horse mackerel</u>	<u>Ie</u>
<u>Centrarchidae</u>	<u><i>Ambloplites rupestris</i></u>	<u>Rock bass</u>	<u>IVb</u>
	<u><i>Lepomis gibbosus</i></u>	<u>Pumpkinseed</u>	<u>IVb</u>
	<u><i>Lepomis macrochirus</i></u>	<u>Bluegill</u>	<u>IV, IVb</u>
	<u><i>Micropterus dolomieu</i></u>	<u>Smallmouth bass</u>	<u>IVb</u>
	<u><i>Micropterus salmoides</i></u>	<u>Largemouth bass</u>	<u>IVb</u>
	<u><i>Pomoxis nigromaculatus</i></u>	<u>Black crappie</u>	<u>IVb</u>
<u>Clupeidae</u>	<u><i>Alosa immaculata</i></u>	<u>Pontic shad</u>	<u>Ie</u>
	<u><i>Sardina pilchardus</i></u>	<u>Pilchard</u>	
	<u><i>Clupea harengus</i></u>	<u>Atlantic herring</u>	<u>IIb, III</u>
	<u><i>Clupea pallasii pallasii</i></u>	<u>Pacific herring</u>	<u>IVa</u>
	<u><i>Dorosoma cepedianum</i></u>	<u>American gizzard shad</u>	<u>IVb</u>
	<u><i>Sardinops sagax</i></u>	<u>South American pilchard</u>	<u>IVa</u>
	<u><i>Sprattus sprattus</i></u>	<u>European sprat</u>	<u>IIb</u>
<u>Cyclopteridae</u>	<u><i>Cyclopterus lumpus</i></u>	<u>Lumpfish</u>	<u>IVd</u>
<u>Cyprinidae</u>	<u><i>Danio rerio</i></u>	<u>Zebra fish</u>	<u>IVa</u>
	<u><i>Notropis hudsonius</i></u>	<u>Spottail shiner</u>	<u>IVb</u>
	<u><i>Notropis atherinoides</i></u>	<u>Emerald shiner</u>	<u>IVb</u>
	<u><i>Pimephales notatus</i></u>	<u>Bluntnose minnow</u>	<u>IVb</u>

Annex 6 (contd)

<u>Family</u>	<u>Scientific name</u>	<u>Common name</u>	<u>Genotype</u>
<u>Cyprinidae</u>	<u><i>Pimephales promelas</i></u>	Fathead <u>Minnow minnow</u>	<u>IVb</u>
<u>Embiotocidae</u>	<u><i>Cymatogaster aggregata</i></u>	<u>Shiner perch</u>	<u>IVa</u>
<u>Engraulidae</u>	<u><i>Engraulis encrasicolus</i></u>	<u>European anchovy</u>	<u>Ie</u>
<u>Esocidae</u>	<u><i>Esox lucius</i></u>	<u>Northern pike</u>	<u>IVb</u>
	<u><i>Esox masquinongy</i></u>	<u>Muskellunge</u>	<u>IVb</u>
<u>Fundulidae</u>	<u><i>Fundulus heteroclitus</i></u>	<u>Mummichog</u>	<u>IVc</u>
<u>Gadidae</u>	<u><i>Gadus macrocephalus</i></u>	<u>Pacific cod</u>	<u>IVa</u>
	<u><i>Gadus morhua</i></u>	<u>Atlantic cod</u>	<u>Ib, III</u>
	<u><i>Merlangius merlangus</i></u>	<u>Whiting</u>	<u>Ie</u>
	<u><i>Micromesistius poutassou</i></u>	<u>Blue <u>Whiting whiting</u></u>	<u>Ib, III</u>
	<u><i>Trisopterus esmarkii</i></u>	<u>Norway pout</u>	<u>Ib, III</u>
<u>Gasterosteidae</u>	<u><i>Gasterosteus aculeatus</i></u>	<u>Three-spine stickleback</u>	<u>IVc</u>
<u>Gobiidae</u>	<u><i>Neogobius melanostomus</i></u>	<u>Round goby</u>	<u>IVb</u>
	<u><i>Pomatoschistus minutus</i></u>	<u>Sand goby</u>	<u>Ib</u>
<u>Ictaluridae</u>	<u><i>Ictalurus Ameiurus nebulosus</i></u>	<u>Brown bullhead</u>	<u>IVb</u>
<u>Labridae</u>	<u><i>Centrolabrus exoletus</i></u>	<u>Rock cook wrasse</u>	<u>III</u>
	<u><i>Ctenolabrus rupestris</i></u>	<u>Goldsinny wrasse</u>	<u>III</u>
	<u><i>Labrus bergylta</i></u>	<u>Ballan wrasse</u>	<u>III</u>
	<u><i>Labrus mixtus</i></u>	<u>Cuckoo wrasse</u>	<u>III</u>
	<u><i>Symphodus melops</i></u>	<u>Corkwing wrasse</u>	<u>III</u>
<u>Lotidae</u>	<u><i>Gaidropsarus vulgaris</i></u>	<u>Three-bearded rockling</u>	<u>Ie</u>
<u>Moronidae</u>	<u><i>Morone americana</i></u>	<u>White <u>Perch perch</u></u>	<u>IVb</u>
	<u><i>Morone chrysops</i></u>	<u>White <u>Bass bass</u></u>	<u>IVb</u>
	<u><i>Morone saxatilis</i></u>	<u>Striped bass</u>	<u>IVb, IVc</u>
<u>Mullidae</u>	<u><i>Mullus barbatus</i></u>	<u>Red mullet</u>	<u>Ie</u>
<u>Osmeridae</u>	<u><i>Thaleichthys pacificus</i></u>	<u>Eulachon</u>	<u>IVa</u>
<u>Percidae</u>	<u><i>Sander vitreus</i></u>	<u>Walleye</u>	<u>IVb</u>
	<u><i>Perca flavescens</i></u>	<u>Yellow perch</u>	<u>IVb</u>
<u>Petromyzontidae</u>	<u><i>Lampetra fluviatilis</i></u>	<u>River lamprey</u>	<u>II</u>
<u>Pleuronectidae</u>	<u><i>Limanda limanda</i></u>	<u>Common dab</u>	<u>Ib</u>
	<u><i>Platichthys flesus</i></u>	<u>European flounder</u>	<u>Ib</u>
	<u><i>Pleuronectes platessus</i></u>	<u>European plaice</u>	<u>III</u>
<u>Rajidae</u>	<u><i>Raja clavata</i></u>	<u>Thornback ray</u>	<u>Ie</u>

Annex 6 (contd)

<u>Family</u>	<u>Scientific name</u>	<u>Common name</u>	<u>Genotype</u>
<u>Salmonidae</u>	<u><i>Coregonus artedii</i></u>	<u>Lake cisco</u>	<u>IVb</u>
	<u><i>Coregonus clupeaformis</i></u>	<u>Lake whitefish</u>	<u>IVb</u>
	<u><i>Coregonus lavaretus</i></u>	<u>Common whitefish</u>	<u>Ia</u>
	<u><i>Oncorhynchus kisutch</i></u>	<u>Coho salmon</u>	<u>IVa</u>
	<u><i>Oncorhynchus mykiss</i></u>	<u>Rainbow trout</u>	<u>Ia-e, III, IVb</u>
	<u><i>Oncorhynchus mykiss X Oncorhynchus kisutch hybrids</i></u>	<u>Rainbow trout X coho salmon hybrids</u>	<u>Ia</u>
	<u><i>Oncorhynchus tshawytscha</i></u>	<u>Chinook salmon</u>	<u>IVa, IVb</u>
	<u><i>Salmo marmoratus</i></u>	<u>Marble trout</u>	<u>Ia</u>
	<u><i>Salmo salar</i></u>	<u>Atlantic salmon</u>	<u>Ia, Ib, II, III, IVa</u>
	<u><i>Salmo trutta</i></u>	<u>Brown trout</u>	<u>Ia, Ib</u>
	<u><i>Salvelinus namaycush</i></u>	<u>Lake trout</u>	<u>Ia, IVa, IVb</u>
	<u><i>Thymallus thymallus</i></u>	<u>Grayling</u>	<u>I</u>
<u>Scophthalmidae</u>	<u><i>Scophthalmus maximus</i></u>	<u>Turbot</u>	<u>Ib, III</u>
<u>Sciaenidae</u>	<u><i>Aplodinotus grunniens</i></u>	<u>Freshwater drum</u>	<u>IVb</u>
<u>Scombridae</u>	<u><i>Scomber japonicus</i></u>	<u>Pacific Chub mackerel</u>	<u>IVa</u>
<u>Soleidae</u>	<u><i>Solea senegalensis</i></u>	<u>Senegalese sole</u>	<u>III</u>
<u>Uranoscopidae</u>	<u><i>Uranoscopus scaber</i></u>	<u>Atlantic stargazer</u>	<u>Ie</u>

[...]

GLOSSARY

EU comment

The EU thanks the OIE and supports the proposed changes to the Glossary.

AQUATIC ANIMAL WASTE

means ~~the entire carcass of an anything generated from aquatic animals, its that have died, have been killed for disease control purposes, or have been killed and processed for human consumption or other purposes. This may include the entire carcass of aquatic animals, their parts, or associated liquids which are intended for disposal.~~

AQUATIC ANIMAL PRODUCTS

means non-viable aquatic animals, parts of aquatics animals, or manufactured goods containing any material derived from and products from aquatic animals that are intended for sale or trade.

VECTOR

means any living organism, other than susceptible species, that transports a *pathogenic agent* to a population of susceptible species ~~susceptible aquatic animal~~ or its food or immediate surroundings. ~~The pathogenic agent may or may not pass through a development cycle within the vector.~~

Proposed amendments of the term ‘waste’ to ‘aquatic animal waste’ in the Aquatic Code

EU comment

The EU supports the proposed amendments of the term ‘waste’ to ‘aquatic animal waste’.

Article	Page	Proposed change
User’s guide, C. Specific issues, 7), last sentence		The assessment for inclusion of aquatic animal products in these articles is based on the form and presentation of the product, the expected volume of waste <u>aquatic animal waste</u> tissues generated by the consumer and the likely presence of viable pathogenic agent in the waste <u>aquatic animal waste</u> .
2.1.4., 2.c), last point		- waste <u>Aquatic animal waste</u> disposal practices
4.2.3., 2.i)		i) disposal of waste <u>aquatic animal waste</u> ;
4.3.6.	60	These conditions include a high level of disease risk (due to the significance of the disease), high pathogen loading, potential high volumes of infected aquatic animals and waste <u>aquatic animal waste</u> , large areas requiring disinfection and large volumes of contaminated water.
4.7.1.	71	The objective of this chapter is to provide guidance on storage, transport, disposal and treatment of <u>aquatic animal wastes</u> so as to manage risks to aquatic animal health.
4.7.2.	71	The scope of this chapter covers <u>aquatic animal waste</u> waste derived from: i) routine aquaculture operations; ii) on shore processing, irrespective of origin; iii) mass killing for disease control purposes and iv) mass mortality (including in the wild).
4.7.3.	71	<u>For the purpose of this chapter:</u> Aquatic animal waste means the entire body or parts of aquatic animals that have died or have been killed for disease control purposes as well as slaughtered aquatic animals, and their parts, that are not intended for human consumption. High risk waste means <u>aquatic animal waste</u> waste that constitutes, or is suspected of constituting, a serious health risk to <u>aquatic animals</u> or humans. Low risk waste means <u>aquatic animal waste</u> waste that is not high risk waste.
4.7.4.	71	The Competent Authority should oversee the efficient and effective disposal of <u>aquatic animal waste</u> waste . [...]: 1) physical, logistical and data access by relevant personnel, in cooperation with stakeholders, including access of the <i>Competent Authority</i> to the <u>aquatic animal waste</u> waste ; 2) movement controls and the authority to make exemptions under certain <i>biosecurity</i> conditions, for example for transport of <u>aquatic animal waste</u> waste to another location for disposal;

Annex 8 (contd)

4.7.5.	72	<p>Following collection, <i>aquatic animal</i> waste <u>waste</u> should be stored for the minimum time practical; however, where storage is necessary there should be sufficient capacity for the expected waste <u>aquatic animal waste</u> and the <i>Competent Authority</i> may require additional measures.</p> <p>[...]</p> <p>The containers of stored <i>aquatic animal</i> waste <u>waste</u> should be leak-proof and secured to prevent contact with <i>aquatic animals</i>, other animals or birds and unauthorised personnel.</p> <p><i>Aquatic animal</i> waste <u>waste</u> infected by an agent causing a <i>disease</i> referred to in the <i>Aquatic Code</i> or suspected of being so, may not be transported without permission from the <i>Competent Authority</i>. [...].</p> <p>Containers used for transport of <i>aquatic animal</i> waste <u>waste</u> should be leak-proof and labelled regarding content. [...]</p>
4.7.6.	72	<p><u>Requirement for approval</u></p> <p>All disposal plants dealing with <i>aquatic animal</i> waste <u>waste</u> should be approved by the <i>Competent Authority</i>. [...]</p> <p><u>Conditions for approval</u></p> <p>For a disposal plant to be approved to deal with <i>aquatic animal</i> waste <u>waste</u>, it should:</p> <p>[...]</p> <p>fulfil requirements for handling the <i>aquatic animal</i> <u>waste</u> and products specified by the <i>Competent Authority</i>. [...]</p> <p><u>Operating requirements</u></p> <p>[...]</p> <p>handling and treatment of <i>aquatic animal</i> waste <u>waste</u> should take place as soon as possible after being received;</p> <p>[...]</p>
4.7.7.	73	<p><u>Rendering</u></p> <p>[...]</p> <p>The process typically involves pre-heating to 50–60°C, followed by cooking of the raw <i>aquatic animal</i> <u>waste</u> at 95–100°C for 15 to 20 minutes. [...]</p> <p><u>Incineration</u></p> <p>[...] Mobile air curtain incinerators enable the process to be carried out on site thus removing the need to transport the <i>aquatic animal</i> <u>waste</u>.</p> <p>Incinerators may only be capable of handling limited volumes of <i>aquatic animal</i> waste <u>waste</u>.</p>
4.7.7.	74	<p><u>Ensiling</u></p> <p>[...]</p> <p>Ensiling of <i>aquatic animal</i> waste <u>waste</u> in an organic acid such as formic acid is an effective method of inactivating most <i>pathogenic agents</i> within 48 hours. [...]</p>

4.7.7.	74	<p><u>Burial</u></p> <p>[...]</p> <p>Whenever possible, the <i>aquatic animal waste</i> waste <u>waste</u> should be subjected to a treatment that ensures inactivation of the <i>pathogenic agents</i> prior to burial.</p> <p>In selecting an acceptable burial site, consideration should be given to the following:</p> <p>[...]</p> <p>Access – easy access for equipment and delivery of <i>aquatic animal waste</i> waste <u>waste</u>. Fencing and restricted admittance may be necessary.</p> <p>Pit construction – [...]Pit dimensions depend on the volume of the <i>aquatic animal waste</i> waste <u>waste</u> to be buried and should be easy to fill.</p> <p>Pit closure – contents should be covered with unslaked lime (CaO) at a rate of 85 kg per 1,000 kg of <i>aquatic animal waste</i> waste <u>waste</u> to hasten decomposition and prevent scavenging.</p> <p><u>Pyre-burning</u></p> <p>Pyre-burning may not be suitable for large amounts of <i>aquatic animal waste</i> waste <u>waste</u>.</p> <p>[...]</p> <p>Access – for equipment to construct the pyre and maintain the fire, for the delivery of fuel and <i>aquatic animal waste</i> waste <u>waste</u>.</p> <p>[...] If the pyre-burning is carried out correctly, <i>aquatic animal wastes</i> waste <u>waste</u> will be destroyed within 48 hours.</p>
4.7.8.	75	<p><u>1. Ensiling</u></p> <p>Ensiling of <i>aquatic animal waste</i> waste <u>waste</u> in an organic acid such as formic acid is an effective method of inactivating most <i>pathogenic agents</i> within 48 hours.</p>
5.4.2.	93	<p>[...] The criteria for inclusion of <i>aquatic animal products</i> in point 1 of Article X.X.11. (mollusc disease-specific chapters), Article X.X.12. (amphibian, crustacean and fish disease-specific chapters) and Article 10.4.16. include consideration of the form and presentation of the product, the expected volume of waste <i>aquatic animal waste</i> tissues generated by the consumer and the likely presence of viable <i>pathogenic agent</i> in the waste <i>aquatic animal waste</i>.</p> <p>[...]</p> <p>It is assumed that: (i) the <i>aquatic animal products</i> are used for human consumption only; (ii) waste <i>aquatic animal waste</i> may not always be handled in an appropriate manner that mitigates the introduction of the <i>pathogenic agent</i>; the level of risk is related to the waste <i>aquatic animal waste</i> disposal practices in each Member's country or territory; [...]</p>

Annex 8 (contd)

5.4.2.	93	<p>Criteria</p> <p>[...]</p> <p>EITHER</p> <p>2) it includes an amount of raw waste <u>aquatic animal waste</u> tissues generated by the consumer that is unlikely to result in the introduction and establishment of the <i>pathogenic agent</i>;</p> <p>OR</p> <p>3) the <i>pathogenic agent</i> is not normally found in the waste <u>aquatic animal waste</u> tissues generated by the consumer.</p>
6.5.3.	129	<p><u>Entry assessment</u></p> <p>[...]</p> <p>- data on trends and occurrence of resistant microorganisms obtained through <i>surveillance</i> of <i>aquatic animals</i> and <i>aquatic animal products</i> and waste <u>aquatic animal waste products</u>.</p> <p>4. <u>Exposure assessment</u></p> <p>[...]</p> <p>disposal practices for waste <u>aquatic animal waste</u> and the likelihood for human exposure to resistant microorganisms or resistance determinants through those waste <u>aquatic animal waste products</u>;</p> <p>[...]</p>

Model articles for declaration of freedom

EU comment

The EU in general supports the proposed changes to this model article.

Comments are inserted in the text below.

Note: time periods in these model articles would be determined by the Aquatic Animals Commission for each disease based on criteria that will be included in a revised Chapter 1.4. For this reason, periods are shown as [X] to indicate that the period is to be determined for each specific disease. Where a period is shown (e.g. ‘the last [two] years’) this indicates an intended default period that may vary depending on the circumstances of each disease.

Article X.X.4.

[Note: new article to outline general requirements for making a declaration of freedom for a country, zone or compartment.]

Requirements for declaration of freedom from [PATHOGEN X]

A Member Country may make a *self-declaration of freedom* from infection with [PATHOGEN X] for the entire country, a *zone* or a *compartment* in accordance with the provisions of Articles X.X.5. to X.X.8., as relevant. The self-declaration must be made in accordance with other relevant requirements of the *Aquatic Code* including that the Member Country:

- 1) complies with the provisions of Chapter 3.1. on the quality of the *Aquatic Animal Health Services*; and
- 2) uses appropriate methods of diagnosis, as recommended in the *Aquatic Manual*; and
- 3) meets all requirements of Chapter 1.4. that are relevant to the self-declaration of freedom.

Article X.X.5.

[Note: equivalent to existing Article X.X.4.]

Country free from infection with [PATHOGEN X]

If a country shares a *zone* with one or more other countries, it can only make a *self-declaration of freedom* from infection with [PATHOGEN X] if the shared water bodies are within countries or *zones* declared free from infection with [PATHOGEN X] (see Article X.X.6.).

EU comment

We believe the entity which should be mentioned at the beginning of the sentence above should be ‘water bodies’ not ‘zone’ i.e. the sentence should read as follows:

“If a country shares a zone water body with ~~one or more~~ other countries, it can only make a *self-declaration of freedom* from infection with [PATHOGEN X] if when those other countries or zones thereof, in which the shared water body is located, are also are within countries or zones declared free from infection with [PATHOGEN X] (see Article X.X.6.).”

As described in Article 1.4.X., a Member Country may make a *self-declaration of freedom* from infection with [PATHOGEN X] for its entire *territory* if:

- 1) none of the susceptible species referred to in Article X.X.2. are present and *basic biosecurity conditions* have been continuously met for at least the last [two] years;

EU comment

For a self-declaration, we consider that two key requirements should be included. First, there is a need to have documentary evidence that none of the susceptible species are present and second, that an assessment of the likelihood of introduction of a species which is susceptible to the pathogen in question has been carried out, and has been assessed to be negligible.

For this reason, we suggest the inclusion of these two requirement in bullet point 1 above to read

- “1) documentary evidence indicates that none of the susceptible species referred to in Article X.X.2. are present, and that the likelihood of introduction has been assessed to be negligible and *basic biosecurity conditions* have been continuously met for at least the last [two] years;”**

OR

- 1) there has been no occurrence of infection with [PATHOGEN X] for at least the last [ten] years, and:
- a) the Member Country can demonstrate that conditions are conducive to the clinical expression of infection with [PATHOGEN X], as described in the corresponding chapter of the *Aquatic Manual*; and
 - b) *basic biosecurity conditions* as described in Chapter 1.4. have been continuously met for at least the last [ten] years;

EU comment

We recommend the inclusion of two extra bullet points to confirm that vaccination has not taken place and that the disease is not present in wild animals. These two bullet points could be as follows:

“c) vaccination against the disease in question has not been permitted;

d) that the disease in question is not known to be established in wild animals of listed species within the Member Country;”

OR

- 3) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [two] years without detection of [PATHOGEN X], and:
- a) *basic biosecurity conditions* have been continuously met from at least [one] year prior to commencement of targeted surveillance;

OR

- 4) it previously made a *self-declaration of freedom* from infection with [PATHOGEN X] and subsequently lost its free status due to the detection of [PATHOGEN X] but the following conditions have been met:
- a) on detection of [PATHOGEN X], the affected area was declared an *infected zone* and a *protection zone* was established; and

- b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of [PATHOGEN X], and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

EU comment

After the detection of a pathogen, we suggest including ‘cleaning’ and ‘fallowing’ as key practices to succeed with the elimination of the pathogen. Based on this, bullet point b) above could read as follows:

“b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of [PATHOGEN X], and the appropriate cleaning and disinfection procedures (as described in Chapter 4.3.) have been completed, followed by fallowing for an appropriate period of time; and”

- c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with [PATHOGEN X]; and
- d) *targeted surveillance*, as described in Chapter 1.4., has been in place for i) at least the last [two] years without detection of [PATHOGEN X] or ii) at least the last [one] year without detection of [PATHOGEN X] if affected farms were not epidemiologically connected to wild populations of *susceptible species*.

EU comment

We believe that a new bullet point between c) and d) above should mention ‘restocking’ and stating that it has to be done from an approved pathogen free source.

In the meantime, part or all of the country, apart from the *infected* and *protection zones*, may be declared a free zone provided that such a part meets the conditions in point 2 of Article X.X.6.

Eu comment

We believe that a free zone should be geographically defined. The above paragraph could reflect this to read:

In the meantime, part or all of the country, apart from the *infected* and *protection zones*, may be declared a free zone provided that such a part is geographically defined and meets the conditions in point 2 of Article X.X.6.

Article X.X.6.

[Note: new article for zone freedom modified from existing Article X.X.5.]

Zone free from infection with [PATHOGEN X]

If a *zone* extends over the *territory* of more than one country, it can only be declared a *zone* free from infection with [PATHOGEN X] if all of the relevant *Competent Authorities* confirm that all relevant conditions have been met.

As described in Article 1.4.X., a Member Country may make a *self-declaration of freedom* from infection with [PATHOGEN X] for a *zone* within its *territory* if:

- 1) none of the *susceptible species* referred to in Article 10.6.2. are present and *basic biosecurity conditions* have been continuously met for at least the last [two] years;

EU comment

As per our comment above in Article X.X.5 bullet point 1) we suggest including also here the need for documentary evidence and the assessment of the likelihood of introduction of a species which is susceptible to the pathogen:

For these reasons, we suggest the inclusion of these two requirement in bullet point 1 above to read

“1) documentary evidence indicates that none of the susceptible species referred to in Article 10.6.2. are present and that the likelihood of introduction has been assessed to be negligible and basic biosecurity conditions have been continuously met for at least the last [two] years;”

OR

- 1) there has been no occurrence of infection with [PATHOGEN X] for at least the last [ten] years, and;
 - a) the Member Country can demonstrate that conditions are conducive to the clinical expression of infection with [PATHOGEN X], as described in the corresponding chapter of the *Aquatic Manual*; and
 - b) *basic biosecurity conditions* as described in Chapter 1.4. have been continuously met for the *zone* for at least the last [ten] years;

EU comment

Again, as per article X.X.5, we recommend the inclusion of two extra bullet points to confirm that vaccination has not taken place and that the disease is not present in wild animals. These two bullet points could be as follows:

“c) vaccination against the disease in question has not been permitted;

d) that the disease in question is not known to be established in wild animals of listed species within the Member Country;”

OR

- 3) *targeted surveillance*, as described in Chapter 1.4., has been in place in the *zone* for at least the last [two] years without detection of [PATHOGEN X], and:
 - a) *basic biosecurity conditions* have been continuously met for at least [one] year prior to commencement of *targeted surveillance*;

OR

- 4) it previously made a *self-declaration of freedom* for a *zone* from infection with [PATHOGEN X] and subsequently lost its free status due to the detection of [PATHOGEN X] in the *zone* but the following conditions have been met:
 - a) on detection of [PATHOGEN X], the affected area was declared an *infected zone* and a *protection zone* was established; and
 - b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of [PATHOGEN X], and the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed; and

EU comment

Similarly to our comments above in article X.X5, after the detection of a pathogen, we suggest including ‘cleaning’ and ‘fallowing’ as key practices which are necessary to

succeed with the elimination of the pathogen. Based on this, bullet point b) above could read as follows:

“b) infected populations within the *infected zone* have been killed and disposed of by means that minimise the likelihood of further transmission of [PATHOGEN X], and the appropriate cleaning and disinfection procedures (as described in Chapter 4.3.) have been completed, followed by fallowing for an appropriate period of time; and”

- c) previously existing *basic biosecurity conditions* have been reviewed and modified as necessary and have continuously been in place since eradication of infection with [PATHOGEN X]; and
- d) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last [two] years without detection of [PATHOGEN X].

EU comment

As per our comment in article X.X.5, we believe that a new bullet point between c) and d) above should include mention of ‘restocking’ and stating that it has to be done from an approved free source.

Article X.X.7.

[Note: new article dedicated to compartments]

Compartment free from infection with [PATHOGEN X]

As described in Article 1.4.X., a Member Country may make a *self-declaration of freedom* from infection with [PATHOGEN X] for a *compartment* within its *territory* if:

- 1) *targeted surveillance*, as described in Chapter 1.4., has been in place in the *compartment* for at least the last [two] years without detection of [PATHOGEN X], and:
 - a) *basic biosecurity conditions* have been continuously met for at least [one] year prior to commencement of *targeted surveillance*;

OR

- 2) it previously made a *self-declaration of freedom* for a *compartment* from infection with [PATHOGEN X] and subsequently lost its free status due to the detection of [PATHOGEN X] in the *zone* but the following conditions have been met:
 - a) all *aquatic animals* within the *compartment* have been killed and disposed of by means that minimise the likelihood of further transmission of [PATHOGEN X], the appropriate *disinfection* procedures (as described in Chapter 4.3.) have been completed, and the *compartment* has been fallowed for at least [X] weeks; and

EU comment

Similarly to our comments above in article X.X.5, after the detection of a pathogen and losing the status, we suggest including ‘cleaning’ as a key practice to succeed with the elimination of the pathogen. Based on this, bullet point b) above could read as follows:

“a) all *aquatic animals* within the *compartment* have been killed and disposed of by means that minimise the likelihood of further transmission of [PATHOGEN X], the appropriate cleaning and disinfection procedures (as described in Chapter 4.3.) have been completed, and the *compartment* has been fallowed for at least [X] weeks; and”

- b) previously existing *basic biosecurity conditions*, including the *biosecurity plan*, have been reviewed and modified as necessary and have continuously been in place from the time of restocking with animals from an approved pathogen free source in accordance with the requirements of Articles X.X.9. [*note: existing Article X.X.7.*] and X.X.10 as appropriate; and
- c) *targeted surveillance*, as described in Chapter 1.4., has been in place for at least the last **one** year without detection of [PATHOGEN X].

Article X.X.8.

[*Note: modified from current Article X.X.6.*]

Maintenance of free status

A country or *zone* that is declared free from infection with [PATHOGEN X] following the provisions of point 1 of Articles X.X.5. or X.X.6. (as relevant) may maintain its status as free from infection with [PATHOGEN X] provided that *basic biosecurity conditions* are continuously maintained.

A country or *zone* that is declared free from infection with [PATHOGEN X] following the provisions of point 2 of Articles X.X.5. or X.X.6. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions are conducive to clinical expression of infection with [PATHOGEN X], as described in the corresponding chapter of the *Aquatic Manual*, and *basic biosecurity conditions* are continuously maintained.

EU comment

The paragraph above and below seems to contradict each other in relation to the level of surveillance which is required to maintain disease freedom for zones.

The paragraph above indicates that when a zone is declared free, targeted surveillance in the zone may be discontinued under certain conditions. However, a zone may be in an area with an unknown of infected status.

On the other hand, the paragraph below states that a zone within a country not declared free, should continue with targeted surveillance.

We believe that to avoid this conflicting situation, the paragraph above should not refer to zones and read:

“A country ~~or zone~~ that is declared free from infection with [PATHOGEN X] following the provisions of point 2 of Articles X.X.5. or X.X.6. (as relevant) may discontinue *targeted surveillance* and maintain its free status provided that conditions are conducive to clinical expression of infection with [PATHOGEN X], as described in the corresponding chapter of the *Aquatic Manual*, and *basic biosecurity conditions* are continuously maintained.”

For declared free *zones* or *compartments* within the *territory* of a country not declared free, *targeted surveillance* should be continued at a level determined by the *Aquatic Animal Health Service* on the basis of the likelihood of *infection*.

In all cases where conditions are not conducive to clinical expression of infection with [PATHOGEN X], ongoing *targeted surveillance*, as described in Chapter 1.4., is required at a level that maintains the level of confidence in freedom from infection with [PATHOGEN X] that was required for the initial declaration of freedom.

**ASSESSMENT OF INFECTION WITH DECAPOD IRIDESCENT VIRUS 1 (DIV1)
FOR LISTING IN CHAPTER 1.3. OF THE
AQUATIC ANIMAL HEALTH CODE**

EU comment

The EU thanks the OIE and supports the revised assessment of infection with DIV1.

Overall Assessment

The OIE Aquatic Animal Health Standards Commission (the Commission) assessed infection with decapod iridescent **virus 1** (DIV1) against the criteria for listing aquatic animal diseases in Article 1.2.2. of the *Aquatic Code* and agreed that infection with (DIV1) meets the OIE criteria for listing, notably 1.: International spread of the disease is likely; 2.: At least one country may demonstrate country or zone freedom from the disease; 3.: A precise case definition is available and a reliable means of detection and diagnosis exists, and 4b.: The disease has been shown to affect the health of cultured aquatic animals at the level of a country or a zone resulting in significant consequences e.g. production losses, morbidity or mortality at a zone or country level (see Table 1 below).

Table 1. Summary of assessment of infection with (DIV1)

	Listing criteria						Conclusion
	1	2	3	4a	4b	4c	
Infection with DIV1	+	+	+	NA	+	-	The disease meets the criteria for listing

NA = not applicable.

Background

A novel member of family *Iridoviridae*, named as decapod iridescent virus 1 (DIV1) (ICTV, 2019), with a double-stranded DNA genome **of** about 166K bp (Li *et al.*, 2017; Qiu *et al.*, 2017b), has been identified as the cause of mass mortalities in shrimp, prawn and crayfish production (Xu *et al.*, 2016; Qiu *et al.*, 2017a; Qiu *et al.*, 2019a). ~~Shrimp~~ Infection with DIV1 has so far been detected in red claw crayfish (*Cherax quadricarinatus*) (Xu *et al.*, 2016), white-leg shrimp (*Penaeus vannamei*) (Qiu *et al.*, 2017), giant freshwater prawn (*Macrobrachium rosenbergii*) (Qiu *et al.*, 2019a), red swamp crayfish (*Procambarus clarkia*) (Qiu *et al.*, 2019a), oriental river prawn (*Macrobrachium nipponense*) (Qiu *et al.*, 2019a) and ridgetail white prawn (*Exopalaemon carinicauda*). Two species of crab, Chinese mitten ~~erap~~ **crab** (*Eriocheir sinensis*) and striped shore crab (*Pachygrapsus crassipes*) have been shown to become infected with DIV1 in experimental challenge through unnatural pathways (Pan *et al.*, 2017). The Commission has recognised the potential significance of infection with DIV1 to many countries given the worldwide importance of crustacean farming and trade. At the moment, infection with DIV1 is considered an “emerging disease” and, as such, should be reported in accordance with Article 1.1.4. of the *Aquatic Code*.

Criteria for listing an aquatic animal disease (Article 1.2.2.)

Criterion No. 1. International spread of the pathogenic agent (via aquatic animals, aquatic animal products, vectors or fomites) is likely.

Assessment

The virus has been detected by PCR or nested PCR method in white-leg shrimp (*P. vannamei*), giant freshwater prawn (*M. rosenbergii*), red swamp crayfish (*P. clarkia clarkii*), oriental river prawn (*M. nipponense*) and ridgetail white prawn (*E. carinicauda*) in farms in China (People’s Rep. of) (Xu *et al.*, 2016; Qiu *et al.*, 2017a; Qiu *et al.*, 2018b; Qiu *et al.*, 2019b). Historically, *P. vannamei* and other susceptible crustacean species have been traded internationally as broodstock and postlarvae for production in new geographic regions, **and *P. vannamei* products are traded internationally.** Thus, **the potential of pathways for transmission are present and** international spread is likely. Histopathology, visualization under TEM and *in-situ* hybridisation provide evidence that the virus can be

found in haematopoietic tissue, gills, hepatopancreas, periopods and muscle (Qiu *et al.*, 2017a). Quantitative PCR detection in experimentally infected shrimp showed that haemolymph and haemopoietic tissues had the highest DIV1 load and muscle tissues had the lowest load (Qiu *et al.*, 2018a; Qiu *et al.*, 2019a).

UNOFFICIAL VERSION

Annex 10 (contd)Conclusion

The criterion is met.

AND

Criterion No. 2. At least one country may demonstrate country or zone freedom from the disease in susceptible aquatic animals.

Assessment

Currently, infection with DIV1 has only been detected in China (People's Rep. of) but the **geographic** distribution of the virus may be wider ~~than what has been reported~~ if mortality events have not been investigated. However, because of the broad distribution of *P. vannamei*, *M. rosenbergii*, and other susceptible species to infection with DIV1, as well as extensive trade in these species, and likely expression of clinical disease and mortality, it is expected that the disease would have been reported elsewhere if the virus had spread widely.

In addition, the disease has been listed as a notifiable disease by the Network of Aquaculture Centres in Asia-Pacific (NACA) in its 'Quarterly Aquatic Animal Disease report' (Asia and Pacific Region) since January 2019. It is **likely**, therefore, **likely** that **the disease** at least one country may **be able to** demonstrate country or zone freedom from the disease in susceptible aquatic animals.

Conclusion

The criterion is met.

AND

Criterion No. 3. A precise case definition is available, and a reliable means of detection and diagnosis exists.

Assessment

Infected *P. vannamei* exhibit empty stomach and guts in all diseased shrimp, slight loss of colour on the surface and in the area of the hepatopancreas, and soft shell. In some individuals slight reddening of the body is observed. Moribund shrimp lose their swimming ability and sink to the bottom of the pond (Qiu *et al.*, 2017a). Diseased *M. rosenbergii* exhibit a white triangle inside the carapace at the base of rostrum which is the location of hematopoietic tissue (Qiu *et al.*, 2019a).

To date, a nested PCR method (Qiu *et al.*, 2017a), a TaqMan probe based real-time PCR (TaqMan qPCR) method (Qiu *et al.*, 2018a), **and an** *in situ* hybridization method (Qiu *et al.*, 2017a) and an *in situ* DIG-labeling-loop-mediated DNA amplification (ISDL) method (Chen *et al.*, 2019) have been published and are available for DIV1 detection. The PCR primers and TaqMan probe have been shown to be specific for DIV1 (no cross-reaction with other shrimp pathogens), with a low detection limit (4 copies per reaction) and high sensitivity and specificity (95.3% and 99.2%, respectively). Validation of the nested PCR method and TaqMan probe based real-time PCR method has occurred.

It can be concluded that **a) reliable means of detection and diagnosis is are** available, and **b) a precise case definition can be developed** based on clinical signs and **e) the use of the available diagnostic tests can be developed**.

Conclusion:

Criterion is met.

AND

Criterion No. 4. a. Natural transmission to humans has been proven, and human infection is associated with severe consequences.

Assessment:

No available data to assess.

Conclusion

Criterion not applicable.

OR

Criterion No. 4.b. The disease has been shown to affect the health of cultured aquatic animals at the level of a country or a zone resulting in significant consequences e.g. production losses, morbidity or mortality at a zone or country level.

Assessment

High mortality (>80%) ~~have~~ has been observed in affected *P. vannamei* and *M. rosenbergii* populations in farms in China (People's Rep. of) (Qiu *et al.*, 2017a; Qiu *et al.*, 2019a). Experimental infection trials mimicking the natural infection pathway (*per os* ~~and reverse garvage~~) in *P. vannamei* ~~has~~ have shown 100% cumulative mortality within 2 weeks (Qiu *et al.*, 2017a). Injection challenges in *P. vannamei*, *C. quadricarinatus*, and *P. clarkii* also ~~exhibited~~ resulted in 100% cumulative mortalities (Xu *et al.*, 2016; Qiu *et al.*, 2017a). Since 2014, some disease events with massive losses of *P. vannamei* and *M. rosenbergii* in coastal provinces of China (People's Rep. of) have been associated with infection with DIV1 (Qiu *et al.*, 2017a). Targeted surveillance in China in 2017 and 2018 ~~revealed that detected~~ has been detected in 11 of 16 provinces (Qiu *et al.*, 2018b; Qiu *et al.*, 2019b). Losses are significant at a country level.

Conclusion

Criterion is met.

OR

Criterion No. 4.c. The disease has been shown to, or scientific evidence indicates that it would affect the health of wild aquatic animals resulting in significant consequences e.g. morbidity or mortality at a population level, reduced productivity or ecological impacts.

Assessment

Infection with DIV1 has been shown to have a significant effect on the health of cultured shrimp, ~~or~~ or crayfish, ~~or~~ or lobsters resulting in significant consequences including morbidity and mortality. It is possible that the disease would affect wild aquatic animals; however, there are no available data to demonstrate impact (e.g. morbidity or mortality) of the disease on wild aquatic animals at a population level.

Conclusion

Criterion is not met.

Annex 10 (contd)**References**

CHEN, X., QIU, L., WANG, H.L. (2019). Susceptibility of *Exopalaemon carinicauda* to the infection with Shrimp hemocyte iridescent virus (SHIV 20141215), a strain of Decapod iridescent virus 1 (DIV1). *Viruses*, **11(4)**: 387. doi: 10.3390/v11040387.

ICTV. (2019). One New Genus with One New Species in the Subfamily Betairidovirinae. Available online: https://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/animal-dna-viruses-and-retroviruses/8051.

LI, F., XU, L. & YANG, F. (2017). Genomic characterization of a novel iridovirus from redclaw crayfish *Cherax quadricarinatus*: evidence for a new genus within the family *Iridoviridae*. *Journal of General Virology*, **98(10)**.

PAN, C. K., YUAN, H. F., WANG, T. T., YANG, F., CHEN, J. M. (2017). Study of *Cherax quadricarinatus* iridovirus in two crab. *Journal of Applied Oceanography*, 36(1): 82-86 (in Chinese).

QIU, L., CHEN, M. M. & WAN, X. Y., (2017a). Characterization of a new member of *Iridoviridae*, Shrimp hemocyte iridescent virus (SHIV), found in white leg shrimp (*Litopenaeus vannamei*). *Scientific Reports*, **7(1)**:11834.

QIU, L., CHEN, M. M. & WANG, R. Y., (2017b). Complete genome sequence of shrimp hemocyte iridescent virus (SHIV) isolated from white leg shrimp, *Litopenaeus vannamei*. *Archives of Virology*, **130(9)**, 1-5.

QIU, L., CHEN, M. M. & WAN, X. Y. (2018a). Detection and quantification of Shrimp hemocyte iridescent virus by TaqMan probe based real-time PCR. *Journal of Invertebrate Pathology*, **154**: 95-101

QIU, L., DONG, X., WAN, X.Y. (2018b). Analysis of iridescent viral disease of shrimp (SHID) in 2017. In *Analysis of Important Diseases of Aquatic Animals in China in 2017*. Fishery and Fishery Administration Bureau under the Ministry of Agriculture and Rural Affairs, National Fishery Technical Extension Center, Eds., China Agriculture Press, Beijing, pp. 187-204, ISBN 978-7-109-24522-8 (in Chinese).

QIU, L., CHEN, X. and ZHAO, R.H. (2019a). Description of a Natural Infection with Decapod Iridescent Virus 1 in Farmed Giant Freshwater Prawn, *Macrobrachium rosenbergii*. *Viruses*, **11(4)**, 354. doi: 10.3390/v11040354.

QIU, L., DONG, X., WAN, X.Y. (2019b). Analysis of iridescent viral disease of shrimp (SHID) in 2018. In *Analysis of Important Diseases of Aquatic Animals in China in 2018*. Fishery and Fishery Administration Bureau under the Ministry of Agriculture and Rural Affairs, National Fishery Technical Extension Center, Eds., (in press) (in Chinese).

XU, L., WANG, T. & LI, F. (2016). Isolation and preliminary characterization of a new pathogenic iridovirus from redclaw crayfish *Cherax quadricarinatus*. *Diseases of Aquatic Organisms*, **120(1)**,17.

CHAPTER 2.3.9.

INFECTION WITH SPRING VIRAEMIA OF CARP VIRUS

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter.

One comment is inserted in the text below.

1. Scope

Infection with spring viraemia of carp virus means infection with the pathogenic agent *Carp sprivivirus* (commonly known as spring viraemia of carp virus [SVCV]), of the Genus *Sprivivirus* and the Family *Rhabdoviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

The virus genome is a non-segmented, negative-sense, single strand of RNA. The genome contains 11,019 nucleotides encoding five proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G) and an RNA-dependent, RNA polymerase (L). The genome does not contain a non-virion (NV) gene between the G and L genes as is found in fish rhabdoviruses of the genus *Novirhabdovirus* (Ahne *et al.*, 2002). The type strain of SVCV is available from the American Type Culture Collection (ATCC VR-1390). Two complete genome sequences of the type strain have been submitted to Genbank (Genbank accession U18101 by Bjorklund *et al.* [1996] and Genbank accession AJ318079 by Hoffmann *et al.* [2002]). The complete genome sequence of isolates from China (People's Rep. of) has also been deposited in Genbank (Genbank accession DQ097384 by Teng *et al.* [2007] and Genbank accession EU177782 by Zhang *et al.* [2009]).

Stone *et al.* (2003) used sequence analysis of a 550 nucleotide region of the G-gene to compare 36 isolates from different fish species and geographical locations that were previously identified by serology as SVCV or pike fry rhabdovirus (PFRV) ~~by serology~~. The analysis showed that the isolates could be separated into four distinct genogroups and that all of the SVCV isolates could be assigned to genogroup I, sharing <61% nucleotide identity with viruses in the other three genogroups. Re-analysis of the sequence data generated for viruses assigned to Genogroup I identified four subgroups (Ia–d). Those viruses originating in Asia were assigned to Subgroup Ia, those from Moldova, the Ukraine and Russia to Subgroups Ib and Ic, and those from the UK to Subgroup Id.

2.1.2. Survival and stability in processed or stored samples

There are limited published data on the stability of the pathogen in host tissues. There is also limited information on the stability of the virus in the tissues after death of a diseased animal. Detection of SVCV in the tissues of recently dead animals by either both reverse transcription polymerase chain reaction (RT-PCR) or and culture may be possible should not be ruled out, and therefore, dead fish ~~as well as moribund should~~ may be taken for analysis if moribund fish are not available.

The virus can be stored for several months when frozen in medium containing 2–5% serum. The virus is most stable at lower temperatures, with little loss of titre ~~for~~ when stored for 1 month at –20°C, or for 6 months at –30 or –74°C (Ahne, 1976; de Kinkelin & Le Berre, 1974). The virus is stable over four freeze (–30°C)–thaw cycles in medium containing 2% serum (de Kinkelin & Le Berre, 1974).

2.1.3. Survival and stability outside the host

The virus has been shown to can remain viable outside the host for 5 weeks in river water at 10°C and for more than 6 weeks in pond mud at 4°C, reducing to 4 days in pond mud at 10°C (Ahne, 1976).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* are: all varieties and subspecies of common carp (*Cyprinus carpio*), bighead carp (*Aristichthys nobilis*), bream (*Abramis brama*), Caspian white fish (*Rutilus kutum*), fathead minnow (*Pimephales promelas*), golden shiner (*Notemigonus crysoleucas*), goldfish (*Carassius auratus*), grass carp (*Ctenopharyngodon idella*), roach (*Rutilus rutilus*) and sheatfish (also known as European or wels catfish) (*Silurus glanis*).

Family	Scientific name	Common name
Cyprinidae	<i>Abramis brama</i>	Bream
	<i>Aristichthys nobilis</i>	Bighead carp
	<i>Carassius auratus</i>	Goldfish
	<i>Ctenopharyngodon idella</i>	Grass carp
	<i>Cyprinus carpio</i>	Common carp (all varieties and subspecies)
	<i>Danio rerio</i>	Zebrafish
	<i>Notemigonus crysoleucas</i>	Golden shiner
	<i>Pimephales promelas</i>	Fathead minnow
	<i>Rutilus kutum</i>	Caspian white fish
Siluridae	<i>Rutilus rutilus</i>	Roach
	<i>Silurus glanis</i>	<u>Sheatfish (also known as European or wels catfish)</u> <u>Wels catfish</u>

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: Crucian carp (*Carassius carassius*), pike (*Esox lucius*), firebelly newt (*Cynops orientalis*), silver carp (*Hypophthalmichthys molitrix*), and yellow perch (*Perca flavescens*) and zebrafish (*Danio rerio*).

Evidence is lacking for these species to either confirm that the identity of the pathogenic agent is SVCV, transmission mimics natural pathways of infection, or presence of the pathogenic agent constitutes an infection.

Family	Scientific name	Common name
Cyprinidae	<i>Carassius carassius</i>	Crucian carp
	<i>Hypophthalmichthys molitrix</i>	Silver carp
Esocidae	<i>Esox lucius</i>	Northern pike
Percidae	<i>Perca flavescens</i>	Yellow perch
Salamandridae	<i>Cynops orientalis</i>	Firebelly newt

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated:

Family	Scientific name	Common name
Catostomidae	<i>Catostomus commersonii</i>	White sucker
Cichlidae	<i>Sarotherodon niloticus</i>	Nile tilapia
	<i>Oreochromis niloticus</i>	
Cyprinidae	<i>Notropis atherinoides</i>	Emerald shiner
	<i>Cirrhinus mrigala</i>	Mrigal carp
	<i>Labeo rohita</i>	Rohu
	<i>Tinca tinca</i>	Tench
Penaeidae	<i>Litopenaeus vannamei</i>	Pacific white shrimp
Salmonidae	<i>Oncorhynchus tshawytscha</i>	Chinook salmon
	<i>Oncorhynchus nerka</i>	Sockeye salmon
	<i>Oncorhynchus mykiss</i>	Rainbow trout
	<i>Oncorhynchus mykiss</i>	<u>Steelhead trout</u>

2.2.3. Non-susceptible species

Species that have been found non-susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Code* are: Largemouth bass (*Micropterus salmoides*), Muskellunge (*Esox masquinongy*), and Walleye (*Sander vitreus*).

Family	Scientific name	Common name
Centrarchidae	<i>Micropterus salmoides</i>	Largemouth bass
Esocidae	<i>Esox masquinongy</i>	Muskellunge
Percidae	<i>Sander vitreus</i>	Walleye

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

Common carp varieties are the principal hosts for SVCV and are considered to be most likely susceptible to be infected with SVCV followed, in order of susceptibility, by other carp species (including hybrids), other susceptible cyprinid species and finally susceptible non-cyprinid fish species. When sampling during surveillance programmes for SVCV, common carp or strains such as koi or ghost (koi x common) carp are preferentially selected, followed by carp hybrids (e.g. common carp x crucian carp), then other carp species such as crucian carp, goldfish, grass carp, bighead carp and silver carp. Should these species not be available then other known susceptible species should be sampled. Cyprinid species may be increasingly mixed together in polyculture systems and the risk of transmission of SVCV between species during disease outbreaks is high (Billard & Berni, 2004).

Generally, young fish up to one-year old are most susceptible to likely to demonstrate clinical signs of disease, but all age groups can be affected. Moreover, there is a high variability in the degree of susceptibility to infection with SVCV among individuals of the same fish species. Apart from the physiological state of the fish, the role of which is poorly understood, age or the age-related status of innate immunity appears to be extremely important to the manifestation of clinical disease: the younger the fish, the higher the susceptibility are more likely to show signs of overt disease, although even adult broodfish can be susceptible to infection.

Fish that have separated from the shoal and found at the water inlet or sides of a pond are more likely to be infected.

2.2.5. Distribution of the pathogen in the host

The transmission of SVCV is horizontal (Fijan, 1988). SVCV appears to enter via the gills and then spreads to the kidney, liver, heart, spleen and alimentary tract. During disease outbreaks high titres of virus occur in the liver and kidney of infected fish, but much lower titres occur in the spleen, gills and brain (Dixon, 2008). The virus has been detected in ovarian fluid (Bekesi & Csontos, 1985), but vertical transmission has yet to be demonstrated.

2.2.6. Aquatic animal reservoirs of infection

Liu *et al.* (2004) isolated SVCV in China (People's Rep. of) from common and koi carp exhibiting no external or internal signs of disease, and similarly, the virus was isolated from apparently healthy wild carp in Canada (Garver *et al.*, 2007). Thus fish surviving infection with SVCV may act as reservoirs of infection.

2.2.7. Vectors

The parasitic invertebrates *Argulus foliaceus* (Crustacea, Branchiura) and *Piscicola geometra* (Annelida, Hirudinea) have been demonstrated to transfer SVCV from diseased to healthy fish under experimental conditions and the virus has been isolated from *A. foliaceus* removed from infected carp (Ahne *et al.*, 2002; Dixon, 2008). It has been demonstrated experimentally that virus can be isolated from fish tissues regurgitated by herons (*Ardea cinerea*) 120 minutes after being fed with SVCV-infected carp, suggesting a potential route for SVCV transmission, but is not known whether such transmission has occurred in nature (Peters & Neukirch, 1986).

Annex 11 (contd)

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

A noticeable increase in mortality will occur in the population during an outbreak of infection with SVCV. Co-infections with koi herpesvirus or carp oedema virus can increase levels of mortality. Disease patterns are influenced by water temperature, age and condition of the fish, population density and stress factors. The immune status of the fish is also an important factor with both nonspecific (e.g. interferon) and specific immunity (serum antibodies, cellular immunity) having important roles. Poor physiological condition of over-wintered fish may be a contributory factor to the onset of clinical disease in infected animals. In European aquaculture, losses can be up to 70% in young carp (Ahne *et al.*, 2002), but are usually from 1 to 40%.

In one survey from Serbia, the virus was isolated by culture in samples collected from 12 of the 38 hatcheries screened over the 10-year period (1992–2002) (Svetlana *et al.*, 2004). The virus occurred sporadically in different ponds on one site, and sporadically from year to year at different sites (Svetlana *et al.*, 2004). In another study, 18 of 30 tissue pools (five fish/pool) of wild, clinically healthy, common carp sampled in Canada in 2006 were positive for SVCV by culture (Garver *et al.*, 2007). The isolation of SVCV in the latter case was from asymptomatic common carp which correlates with this observation suggesting that SVCV infection may often be clinically inapparent (Fijan, 1999).

2.3.2. Clinical signs, including behavioural changes

Fish can become lethargic, separate from the shoal and gather at the water inlet or sides of a pond and some may experience loss of equilibrium. Clinical signs of infection with SVCV are nonspecific and not all fish will exhibit all of the signs. Two of the most obvious and consistent features are abdominal distension and haemorrhages, which may be pale and occur on the skin, fin bases, eyes and gills, which may be pale. The skin may darken and exophthalmia is often observed. The vent may be swollen, inflamed and trail mucoid casts. During an outbreak of infection with SVCV there will be a noticeable increase in mortality in the population. Diseased fish usually appear darker in colour. There may be no clinical signs in cases with a sudden onset of mortality.

2.3.3 Gross pathology

There are no pathognomonic gross lesions. Lesions may be absent in cases of sudden mortality. Gross pathologies are mainly documented for common carp and may include excess ascitic fluid in the abdominal cavity, usually containing blood, degeneration of the gill lamellae and inflammation of the intestine, which contains mucous instead of food. Oedema and haemorrhage of the visceral organs is commonly observed (the spleen is often enlarged), and organs adhere to each other and to the peritoneum. Focal haemorrhages may be seen in the muscle and fat tissue, as well as in the swim bladder (see Dixon, 2008). However, petechial haemorrhages are infrequent uncommon in cases caused by Asian strains of SVCV (Dikkeboom *et al.*, 2004).

2.3.4. Modes of transmission and life cycle

The transmission of SVCV is horizontal (Fijan, 1988). Horizontal transmission may be direct, or via water, fomites or vectors (Section 2.2.7.) (Fijan, 1988). The virus appears to enter the host via the gills. A viraemia follows and the virus rapidly spreads to the liver, kidney, spleen and alimentary tract. The virus can be detected in faeces and is also shed into the water via faeces and urine (Ahne, 1982).

Vertical or and 'egg-associated' transmission cannot be ruled out following one report of isolation of SVCV from carp ovarian fluid, although there have been no further reports (Bekesi & Csontos, 1985).

Horizontal transmission may be direct or vectorial, water being the major abiotic vector (Fijan, 1988). Invertebrate vectors (Section 2.2.6.) and fomites may also be involved in transmission of SVCV (Fijan, 1988). Once SVCV is established in populations, it may be very difficult to eradicate without destroying all susceptible species and vectors types of life at the site.

2.3.5. Environmental and management factors

Disease outbreaks in carp generally occur between 11 and 17°C. They rarely occur below 10°C, and mortalities, particularly in older fish, decline as the temperature exceeds 22°C (Fijan, 1988). However, the virus was isolated from apparently healthy fish from a lake in Canada that had been sampled over a 13-day period during which the water temperature varied between 24.2°C and 27.3°C (Garver *et al.*, 2007). These fish may have been more susceptible to infection as they were penned and detection was during spawning. Secondary and concomitant bacterial and/or parasitic infections can affect the mortality rate and display the appearance of clinical signs. In carp, the disease is often observed during in-spring time (hence the common name for the disease), particularly in countries having cold winters. It is believed that the poor condition of the over-wintered fish may be a contributory factor in the disease occurrence of clinical disease. Clinical The disease can occur in fish in quarantine following the stress of transportation, even though there has been no evidence of infection prior to transportation.

2.3.6. Geographical distribution

For a long time, the geographical range of SVCV was limited to countries of the European continent that experience low water temperatures during winter. Consequently, the disease has been recorded from most European countries, and from certain of the western Independent States of the former Soviet Union (Belarus, Georgia, Lithuania, Moldova, Russia and the Ukraine) (see Dixon 2008 for references to these and the following locations). However, in 1998, the disease was recorded in South America (in goldfish in a lake in Brazil) and in 2002 in the USA, North America, and in 2006 in Canada. The virus was first detected in Asia. Detection of the virus in carp in China (People's Rep. of) was confirmed in 2004.

For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

A safe and effective vaccine is not currently available; however, a number of the efficacy of an experimental DNA vaccine has been investigated. Inactivated preparations, live attenuated vaccines and DNA vaccines have given encouraging results (Dixon, 2008, Emmenegger & Kurath, 2008). The use of live attenuated vaccines or the DNA vaccines might affect diagnostic performance.

2.4.2. Chemotherapy including blocking agents

Methisoprinol inhibits the replication of SVCV *in vitro*, but has not been tested under carp culture conditions (Siwicki *et al.*, 2002).

2.4.3. Immunostimulation

Injection into carp of single-stranded and double-stranded RNA (which is an interferon inducer) protected carp for longer than 3 weeks, but the treatment is not effective by bath administration (Alikin *et al.*, 1996).

2.4.4. Breeding resistant strains

The "Krasnodar" strain of common carp has been bred for increased resistance to SVCV (Kirpichnikov *et al.*, 1993).

2.4.5. Inactivation methods

The virus is inactivated at 56°C for 30 minutes, at pH 12 for 10 minutes and pH 3 for 2 hours (Ahne, 1986). Oxidising agents, sodium dodecyl sulphate, non-ionic detergents and lipid solvents are all effective for inactivation of SVCV. The following disinfectants are also effective for inactivating the virus: 3% formalin for 5 minutes, 2% sodium hydroxide for 10 minutes, 540 mg litre⁻¹ chlorine for 20 minutes, 200–250 ppm (parts per million) iodine compounds for 30 minutes, 100 ppm benzalkonium chloride for 20 minutes, 350 ppm alkyltoluene for 20 minutes, 100 ppm chlorhexidine gluconate for 20 minutes and 200 ppm cresol for 20 minutes (Ahne, 1982; Ahne & Held, 1980; Kiryu *et al.*, 2007).

2.4.6. Disinfection of eggs and larvae

Eggs can be disinfected by iodophor treatment (Ahne & Held, 1980).

2.4.7. General husbandry

Methods to control infection with SVCV rely on avoiding exposure to the virus coupled with good hygiene practices. This is feasible on small farms supplied by spring or borehole water and a secure system to prevent fish entering the farm via the discharge water. Hygiene measures should include disinfection of eggs by iodophor treatment (Ahne & Held, 1980), until it has been confirmed unequivocally that vertical transmission does not occur, regular disinfection of ponds, chemical disinfection of farm equipment, careful handling of fish to avoid stress and safe disposal of dead fish. Reducing fish stocking density during winter and early spring will reduce the spread of the virus. In rearing facilities with a controlled environment, elevation of water temperature above 19–20°C may stop or prevent outbreaks of infection with SVCV.

Annex 11 (contd)

3. Specimen selection, sample collection, transportation and handling

This Section draws on information in Sections 2.2., 2.3. and 2.4. to identify populations, individuals and samples which are most likely to be infected.

3.1. Selection of populations and individual specimens

Sampling samples, target comprise of susceptible species on the site with each group being represented in the sample. A group is defined as a The population to be sampled may be stratified into groups of the same fish species that shares a common water supply and originate from the same broodfish or spawning population. Generally young Moribund fish up to 1 year old are most susceptible to clinical disease, but all age groups can be affected. Any moribund fish present in the fish population to be sampled should be sampled selected first for sample collection and the remainder of the samples should comprise randomly selected live fish from all groups of susceptible species rearing units that represent the lot being examined.

Clinical inspections should be carried out during a period when the water temperature is between 11°C and 17°C. All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention should be paid to the water outlet area where weak fish tend to accumulate due to the water current.

For the purposes of disease surveillance, fish to be sampled are selected as follows:

- i) Common carp or strains such as koi or ghost (koi x common) carp are preferentially selected, followed by carp hybrids (e.g. common carp x crucian carp), then other carp species such as crucian carp, goldfish, grass carp, bighead carp and silver carp. Susceptible species should be sampled proportionally, or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status).
- ii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.
- iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with SVCV should be collected. Ideally fish should be collected while alive, however, recently dead fish can also be selected for diagnostic testing purposes. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time. There may be no clinical signs or gross pathognomonic lesions and no clinical signs in cases of sudden mortality—(see Section 4.1.1.).

3.2. Selection of organs or tissues

Kidney, spleen, gill and encephalon should be selected from subclinically infected fish (apparently healthy fish).

For clinically affected fish: whole fry ~~alevin~~—(body length ≤ 4 cm), entire viscera including kidney and encephalon-brain (> 4 cm body length ≤ 6 cm) or, for larger ~~sized~~ fish, liver, kidney, spleen and encephalon should be selected.

3.3. Samples or tissues not suitable for pathogen detection

Virus isolation may also not be possible from Decomposed clinical samples A number of studies in which attempts were made to isolate virus from reproductive fluids were unsuccessful, although and seminal fluid samples are not suitable. While the virus has been isolated at low frequency from ovarian, but not seminal, fluids, the suitability of these tissues for detection of SVC samples has not been substantiated (Bekesi & Csontos, 1985).

3.4. Non-lethal sampling

Serological assays for antibodies can be undertaken on blood samples; the and can indicate possible exposure to SVCV, however, serology is not a suitable test for making a suspect diagnosis. can only be used for a presumptive diagnosis given cross reactivity of anti-SVCV antibodies with viruses of the species pike fry sprivirus allows for a presumptive indication of infection with SVCV.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.2.0 or 2.3.0 or 2.4.0.

3.5.1. Samples for pathogen isolation

Samples for virus isolation (Section 3.2.) should be transported to the laboratory at 4°C using refrigerated containers or on ice, preferably in virus transport medium and tested within 24 hours or, in exceptional circumstances, 48 hours. The shipment of organ samples is preferred, but live or whole dead fish can be submitted to the testing laboratory if necessary. If this is not possible, samples can be frozen, but there may be loss of virus viability on thawing the samples. Repeated freeze–thawing of the sample must be avoided.

3.5.2. Preservation of Fixed samples for molecular detection

~~Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent grade (absolute) ethanol. The recommended ratio of ethanol to tissue is 10:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. [Alternatives to ethanol can be mentioned if they can be referenced.]~~

The material collected for virus culture is generally used for the molecular diagnostic assays, but additional tissue samples for RT-PCR can be preserved in commercially available RNA preservation solutions according to the manufacturers' recommendations, or, alternatively, samples can be preserved in 80–90% (v/v) analytical grade (absolute) ethanol at the recommended ratio of ethanol to tissue of 10:1.

3.5.3. Fixed samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Histology samples from each individual fish must be **taken placed** into 10% neutral buffered formalin (NBF) immediately after collection to prevent sample deterioration. The recommended ratio of fixative to tissue is 10:1 and each sample should be **cut cleanly and be** no thicker than approximately 4 mm to allow the fixative to penetrate the material ~~and should be cut cleanly.~~

EU Comment:

We have previously commented that ‘In-situ hybridisation’ is not a method that is recommended in Table 4.1. where the OIE includes a list of recommended diagnostic methods and their level of validation for surveillance. The table does not include any assessment of the level of validation for ‘In-situ hybridisation’. For these reasons, we still think that it should therefore be removed from 3.5.3.

3.5.4. Fixed samples for electron microscopy

~~EM sampling is~~ Samples for electron microscopy are not routinely required as standard, and the material is and are collected only when where it is considered beneficial to facilitate potential further diagnostic investigation work. From each fish sampled, a 2 mm cubed (approximately) sample section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.

3.5.5. Samples for other tests

Tubes for the separation of serum are available commercially. After collection, the blood is allowed to clot by leaving it undisturbed at room temperature. This usually takes 15–30 minutes. Serum is clarified by centrifuging at 1000–2000 **g** for 10 minutes in a refrigerated centrifuge at 4–8°C.

It is important to immediately transfer the liquid component (serum) into a clean polypropylene tube using a Pasteur pipette and maintain the samples at 2–8°C while handling. If the serum is not analysed immediately, it should be apportioned into 0.5 ml aliquots, stored, and transported at –20°C or lower. It is important to avoid freeze–thaw cycles because this is detrimental to many serum components. Samples that are haemolysed, icteric or lipaemic can invalidate certain tests.

3.6. Pooling of samples

Traditionally pools of five animals have been used and more recently this has been increased to pools of ten animals for virus culture. However, no published data on the effect of pooling on test characteristics has been published.

Pooling of samples from more than one individual animal for a given purpose should only be recommended where supporting data on diagnostic sensitivity and diagnostic specificity are available. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

+++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;

++ = Suitable method(s) but may need further validation;

+ = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;

Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities, repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of **apparently** healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts												
Histopathology ³												
Cytopathology ³												
Cell or artificial media culture		++	++	1 ₃		++	++	1 ₃		++	++	1 ₃
Real-time PCR												
Conventional PCR		++	++	1 ₂		++	++	1 ₂		++	++	1 ₂
Amplicon sequencing ⁴									+++	+++	1 ₃	
<i>In-situ</i> hybridisation												
<u>Immunohistochemistry</u>						++	++	1				
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA						++	++	1				
<u>IFAT</u> Other antigen detection methods						++	++	1				

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification;

Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay.

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6).

²Early and juvenile life stages have been defined in Section 2.2.4.

³Histopathology and cytopathology can be validated if the results from different operators have been statistically compared.

⁴Sequencing of the PCR product.

Shading indicates the test is inappropriate or should not be used for this purpose.

Annex 11 (contd)**4.1. Wet mounts**

Not applicable.

4.2. Histo pathology and cytopathology

Histopathological changes can be observed in all major organs. In the liver, blood vessels show oedematous perivascularitis progressing to necrosis. Liver parenchyma shows hyperaemia with multiple focal necrosis and degeneration. The heart shows pericarditis and infiltration of the myocardium progressing to focal degeneration and necrosis. The spleen shows hyperaemia with hyperplasia of the reticuloendothelium and enlarged melanomacrophage centres, and the pancreas is inflamed with multifocal necrosis. In the kidney, damage is seen to excretory and haematopoietic tissue. Renal tubules are clogged with casts and the cells undergo hyaline degeneration and vacuolation. The intestine shows perivascular inflammation, desquamation of the epithelium and atrophy of the villi. The peritoneum is inflamed, and lymph vessels are filled with detritus and macrophages. In the swim bladder, the epithelial lamina changes from a monolayer to a discontinuous multi-layer and vessels in the submucosa are dilated with nearby lymphocyte infiltration.

As the histopathological presentation picture is not specific for the disease, and not all fish will exhibit each feature (Misk *et al.*, 2016), microscopic methods by themselves are not recommended for diagnosis of SVC ~~as the histopathological picture is not specific for the disease~~. They may, however, provide supporting evidence, particularly, when immunohistochemistry immunohistological (IHC) or nucleic acid DNA-based *in-situ* hybridisation methods are used (see the relevant Sections below).

~~Fixed sections can also be used for histoimmunochimistry (but see caveats in Section 4.6.).~~

4.3. Cell or artificial media culture for isolation**4.3.1. Cell lines**

~~If culturing viruses~~ The recommended cell lines for SVCV detection are EPC, FHM or GCO. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

4.3.2. Sample preparation and inoculationCell culture

Cell line to be used: EPC, FHM or GCO.

Virus extraction: Use the procedure described in Section A.2.2.2 of Chapter 2.3.0.

Inoculation of cell monolayers: make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium (i.e. the homogenate supernatants will be 1/100 and 1/1000 dilutions of the original organ material) and transfer an appropriate volume of each of these two dilutions on to 24-hour-old cell monolayers drained of their culture medium. Alternatively, make a single tenfold dilution of the 1/10 organ homogenate (i.e. a 1/100 dilution of the original organ material) and add an appropriate volume of both the 1/10 and 1/100 dilutions directly to undrained 24 hour-old cell monolayers, to effect 1/100 and 1/1000 final dilutions of the organ homogenate. Should toxicity of the sample be a problem, make two serial tenfold dilutions of the 1/10 organ homogenate supernatants in cell culture medium as described above and inoculate at least 2 cm² of drained cell monolayer with 100 µl of each dilution. Allow to adsorb for 0.5–1 hour at 10–15°C, withdraw the inoculum and add cell culture medium buffered at pH 7.6 and supplemented with 2% fetal calf serum (FCS) (1 ml well⁻¹ for 24-well cell culture plates). Incubate at 20°C.

Monitoring incubation: Follow the course of infection in positive controls and other inoculated cell cultures by microscopic examination at ×40–100 magnification for 7 days. The use of a phase-contrast microscope is recommended.

Maintain the pH of the cell culture medium at between 7.3. and 7.6. during incubation. This can be achieved by the addition to the inoculated medium of sterile bicarbonate buffer (for tightly closed cell culture flasks) or HEPES-buffered medium (HEPES = N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) or 2 M Tris (Tris [hydroxymethyl] aminomethane)/HCl buffer solution (for cell culture plates).

If a cytopathic effect (CPE) appears in those cell cultures inoculated with the dilutions of the tested homogenate supernatants, identification procedures must be undertaken immediately ~~(see Section 4.6.2).~~

Subcultivation procedures: Using a pipette, try to dislodge cells from the cell culture vessels and collect aliquots of cell culture medium plus cells from all inoculated monolayers, keeping different groups separate. The aliquots of the 1/100 and 1/000 dilutions are pooled and inoculated on to fresh 24 hour-old cell cultures to effect 1/10 and 1/100 final dilutions of the pooled aliquots. Incubate and monitor as described above. ~~If no CPE occurs, the test may be declared negative.~~

If no CPE occurs the test may be declared negative. However, if undertaking surveillance to demonstrate freedom from SVCV it would be advisable to screen the cells at the end of the 14 days using an SVCV-specific RT-PCR ~~or real-time RT-PCR~~ (Section 4.4.). Following a positive result culture should be re-attempted.

Following isolation, the virus must be identified, and this can be achieved by antigen detection methods, virus neutralisation or nucleic acid identification methods. The former two methods are generally regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses occur. Commercially available kits using polyclonal antibodies may also lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). Nucleic acid detection methods must always be followed up by sequencing or use of a method such as reverse hybridisation (Sheppard *et al.*, 2007) to confirm the identity of the virus.

4.4. Nucleic acid amplification

4.4.1. Real-time PCR

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control **if available and validated.**

Real-time RT-PCR assays are available to detect and confirm **infection with** SVCV (Yue *et al.*, 2008; Zhang *et al.*, 2009), however, they are not currently recommended as they have not been sufficiently validated.

4.4.2. Conventional PCR

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~ **Positive and negative controls should be run with each stage of the assays: extraction, RT-PCR and second round PCR. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.**

Nested reverse-transcription polymerase chain reaction (RT-PCR) (confirmation of virus identity from cell culture isolation or directly from fish tissue extracts)

The genome of SVCV consists of a single strand of RNA of approximately 11 kb, with negative polarity. Amplification of a 714 bp fragment of SVCV cDNA is performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' SVCV R2), using a modification of the method of Stone *et al.* (2003).

- i) Total RNA is extracted from 100 µl of supernatant from cell cultures exhibiting CPE or 50 µl of fish tissue extract and dissolved in 40 µl molecular biology grade DNase- and RNase-free water.

A number of total RNA extraction kits are available commercially that will produce high quality RNA suitable for RT-PCR. ~~Examples are Trizol Reagent[®] (RL, Life Technologies, Paisley, UK), SV Total RNA isolation system (Promega) and Nucleospin[®] RNA (AB gene), EZ virus mini kit, Ez RNA tissue mini kit (Qiagen).~~

- ii) For cDNA synthesis, a reverse transcription reaction is performed at 37°C for 1 hour in a 20 µl volume consisting of 1 × M-MLV RT reaction buffer (50 mM Tris, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl₂) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units M-MLV reverse transcriptase (Promega, Southampton, UK) or an equivalent reverse transcriptase system and 1/10 of the total RNA extracted above.

UNOFFICIAL VERSION

Annex 11 (contd)

- iii) PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM MgCl₂, 200 µM dNTPs, 50 pmol each of the SVCV R2 and SVCV F1 primers, 1.25 units of Taq DNA polymerase, and 2.5 µl reverse transcription reaction mix. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (714 bp) is analysed by agarose gel electrophoresis.
- iv) If the CPE in culture is not extensive it is possible that a visible product will not be generated using a single round of amplification. To avoid such problems, use the semi-nested assay using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3' (SVC R4) according to Stone *et al.* (2003).
- v) The second round of PCR is performed in a 50 µl reaction volume 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) containing 2.5 mM MgCl₂, 200 µM dNTPs, 50 pmol each of the SVCV R4 and SVCV F1 primers, 1.25 units Taq DNA polymerase, and 2.5 µl of the first round product. The reaction mix is subjected to 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. Amplified DNA (606 bp) is analysed by agarose gel electrophoresis.
- vi) All amplified products are confirmed as SVCV in origin by sequencing, and the SVCV subtype (Ia-I d) is identified using a BLAST search (<http://www.ncbi.nlm.nih.gov/blast/>) or by phylogenetic analysis using the SVCV sequences available in public sequence databases. Phylogenetic analysis is undertaken using a 426 bp region corresponding to nucleotides 429–855 of the glycoprotein gene.
- vii) In cases where the CPE is extensive and the virus replicates to a high titre, or where a semi-nested RT-PCR assay was used, sufficient PCR amplicon will be available for direct sequencing. Where the amplified product is weak it is recommended that the product be inserted into an appropriate sequencing vector (e.g. pGEM-T, pCR® 4-TOPO®) prior to undertaking the sequencing. At least two independent amplification and sequencing events should be undertaken to eliminate potential sequence errors introduced by the Taq polymerase.

~~The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.~~

~~**NOTE:** The appropriate IUB codes have been used where appropriate and are indicated by an asterisk (*).~~

Reverse-transcription polymerase chain reaction (RT-PCR) (confirmation of virus identity)

Additional conventional RT-PCR assays are available to detect and confirm SVCV infections (Koutna *et al.*, 2003; Shimahara *et al.*, 2016). A generic primer set based on the polymerase gene also identifies viruses from both the *Sprivirus* and *Perhabdovirus* genera and can be used to screen a virus culture (Ruane *et al.*, 2014). With the exception of the conventional PCR assay developed by Shimahara *et al.* (2016) the other assays were not sufficiently fully validated against representatives from each of the recognised SVCV genogroups and they may fail to detect the full range of SVCV genotypes.

A summary of the Shimahara *et al.* (2016) RT-PCR method follows. Amplification of a 369 bp fragment of SVCV glycoprotein gene is performed using primers as follows: SVCV-G1: 5'-TGA-AGA-YTG-TGT-CAA-TCA-AGTC-3' and SVCV-G2: 5'-GCG-ART-GCA-GAG-AAA-AAG-TG-3'. Preparation of RNA template is the same as nested RT-PCR above. Reverse transcription of SVCV RNA and amplification of cDNA are carried out using SuperScript III one-step RT-PCR with PlatinumR Taq (Invitrogen) according to the manufacturer's instructions. The RT-PCR reaction mixture contained 10 pmol of each primer, 12.5 µl of 2x reaction mix, 1 µl of SuperScript III RT/Platinum Taq Mix and 2.5 µl template. After reverse transcription at 50°C for 30 minutes and 94°C for 2 minutes, 40 amplification cycles of 94°C for 15 seconds, 56°C for 30 seconds and 68°C for 1 minute followed by a final extension step at 68°C for 7 minutes is performed. All amplified products are confirmed as SVCV in origin by sequencing.

UNOFFICIAL VERSION

4.4.3. Other nucleic acid amplification methods

Loop-mediated isothermal amplification assays are available to detect and confirm SVCV infections (Shivappa *et al.*, 2008), however, they are currently not recommended as they are not sufficiently validated.

Infection with SVCV has also been confirmed-detected using RT-PCR and hybridisation with non-radioactive probes to determine the genotype (Oreshkova *et al.*, 1999; Sheppard *et al.*, 2007), however, it is currently not recommended as it is not sufficiently validated.

4.5. Amplicon sequencing

~~See above (Section 4.4.2). All Nucleotide sequencing of all RT-PCR amplicons should be sequenced to confirm that they are SVCV in origin (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis.~~ SVCV-specific ~~products-sequences~~ will share a higher degree of nucleotide ~~identity-similarity~~ to one of the published reference sequences for SVCV (Genbank accession U18101, AJ318079, DQ097384 and EU177782) compared to the published reference sequences for the *Pike spriviviruses* (GenBank FJ872827, KC113518 and KC113517).

4.6. ~~In-situ hybridisation (and histoimmunochemistry)~~

~~Although in-situ hybridisation can be used to locate SVCV the virus in different tissues on-in known positive animals, but this assay is currently not recommended as it has not been well-validated as a diagnostic tool for the detection of SVCV as a diagnostic tool.~~

4.7. Immunohistochemistry

SVCV can be detected by immunohistochemistry, however, care must be taken with interpreting the results of ~~serological-these~~ tests for SVCV, and positive results ~~from antibody-based assays~~ should be confirmed by RT-PCR and sequencing (~~see Section 4.8.~~).

- i) Bleed the fish thoroughly.
- ii) Make kidney imprints on cleaned glass slides or at the bottom of the wells of a plastic cell culture plate.
- iii) Store and transport the kidney pieces as indicated in Section 2.2.1. of Chapter 2.3.0. together with the other organs required for virus isolation.
- iv) Allow the imprint to air-dry for 20 minutes.
- v) Fix with cold acetone (stored at -20°C) for glass slides or 80% acetone in water or 30% acetone in ethanol, also at -20°C, for plastic wells. Let the fixative act for 15 minutes. Allow the imprints to air-dry for at least 30 minutes and process immediately or freeze at -20°C.
- vi) Rehydrate the imprints if they have been stored frozen by four rinsing steps with PBS containing 0.05% Tween 20 (PBST), and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.
- vii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.
- viii) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).
- ix) Incubate the imprints with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.
- x) Rinse four times with PBST.

Annex 11 (contd)

- xi) Incubate the imprints with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.
- xii) Rinse four times with PBST.
- xiii) View the treated imprints on plastic plates immediately, or mount the slides with cover-slips using glycerol saline at pH 8.5, or a commercially-available mountant.
- xiv) Examine under incident ultraviolet (UV) light using a microscope with $\times 10$ eye pieces and $\times 20$ or $\times 40$ objective lenses having numerical aperture of >0.65 and >1.3 , respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.8. Bioassay

Not available.

4.9. Antibody-based or antigen-based detection methods (ELISA, etc.)

Serological-Antibody- or antigen-based methods that detect SVCV must be regarded as presumptive unless fully validated monoclonal or polyclonal antibodies are used, as cross reactions with other viruses—closely related spriviviruses (PFRV, GrCRV and TenRV) may occur. Commercially available kits using polyclonal antibodies may lack specificity, and those using monoclonal antibodies may not detect all subgenogroups of SVCV (Dixon & Longshaw, 2005). These techniques should not be used as a screening method.

4.9.1. Antigen enzyme-linked immunosorbent assay (ELISA)

Virus identification by enzyme-linked immunosorbent assay (ELISA)

- i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 ($200 \mu\text{l well}^{-1}$). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.
- ii) Incubate overnight at 4°C .
- iii) Rinse four times with PBST.
- iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C ($300 \mu\text{l well}^{-1}$).
- v) Rinse four times with PBST.
- vi) Add 2% non-ionic detergent (Triton X-100 or Nonidet P-40) to the virus suspension to be identified.
- vii) Dispense $100 \mu\text{l well}^{-1}$ of two- or four-step dilutions of the virus to be identified, and of the non-infected cell culture harvest (negative control). Also include SVCV positive control virus. Incubate for 1 hour at 37°C .
- viii) Rinse four times with PBST.
- ix) Add to the wells, $200 \mu\text{l}$ of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. An MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C .
- x) Rinse four times with PBST.
- xi) If HRPO-conjugated antibody has been used, go to step xiii. Otherwise, add $200 \mu\text{l}$ of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C .

- xii) Rinse four times with PBST.
- xiii) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.

Enzyme-linked immunosorbent assay (ELISA) using tissue homogenates

See Section A-2.2.2 of Chapter 2.3.0, for obtaining organ homogenates.

- i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) specific for SVCV, in 0.02 M carbonate buffer, pH 9.5 (200 µl well⁻¹). Ig may be polyclonal or monoclonal Ig originating most often from rabbit or mouse, respectively. For the identification of SVCV, monoclonal antibodies (MAbs) specific for certain domains of the nucleocapsid (N) protein are suitable.
- ii) Incubate overnight at 4°C.
- iii) Rinse four times with PBST.
- iv) Block with skim milk (5% in carbonate buffer) or other blocking solution for 1 hour at 37°C (300 µl well⁻¹).
- v) Rinse four times with PBST.
- vi) Store a 1/4 aliquot of each homogenate at 4°C, in case the test is negative and virus isolation in cell culture is required.
- vii) Treat the remaining part of the homogenate with 2% Triton X-100 or Nonidet P-40 and 2 mM of phenyl methyl sulphonide fluoride; mix gently.
- viii) Dispense 100 µl well⁻¹ of two- or four-step dilutions of the sample to be identified, and of negative control tissues. Also include an SVCV positive control virus. Incubate for 1 hour at 37°C.
- ix) Rinse four times with PBST.
- x) Add to the wells, 200 µl of horseradish peroxidase (HRPO)-conjugated MAb or polyclonal antibody to SVCV; or polyclonal IgG to SVCV. A MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin can also be used. Incubate for 1 hour at 37°C.
- xi) Rinse four times with PBST.
- xii) If HRPO-conjugated antibody has been used, go to step xiv. Otherwise, add 200 µl of HRPO-conjugated streptavidin or ExtrAvidin (Sigma) to those wells that have received the biotin-conjugated antibody and incubate for 1 hour at 37°C.
- xiii) Rinse four times with PBST.
- xiv) Add 200 µl of a suitable substrate and chromogen, such as tetramethylbenzidine dihydrochloride. Stop the course of the test when positive controls react, and read the results.
- xv) If the test is negative, process the organ samples stored at 4°C, for virus isolation in cell culture as described in Section 4.3.

4.9.2. Indirect fluorescent antibody test (IFAT)

Virus identification Confirmation of virus identity by the indirect fluorescent antibody test (IFAT)

- i) Prepare monolayers of cells in 2 cm² wells of plastic cell culture plates, flasks or on cover-slips or glass slides in order to reach approximately 80% confluency within 24 hours of incubation at 25°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.

Annex 11 (contd)

- ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks. For tests using cells cultured on glass cover-slips or slides, the dilutions are made in sterile containers and then used to inoculate the cells.
- iii) Dilute the control virus suspension of SVCV in a similar way, in order to obtain a virus titre of about 5000–10,000 PFU ml⁻¹ in the cell culture medium.
- iv) Incubate at 20°C for 24 hours.
- v) Remove the cell culture medium, rinse once with 0.01 M phosphate-buffered saline (PBS), pH 7.2, then three times briefly with cold acetone (stored at –20°C) for slides or cover-slips or 80% acetone in water or 30% acetone in ethanol, also at –20°C, for cells on plastic substrates. Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.
- vi) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.
- vii) Rehydrate the dried cell monolayers, if they have been stored frozen, by four rinsing steps with ~~PBS containing 0.05% Tween 20~~ PBST and remove this buffer completely after the last rinse. Block with 5% skim milk or 1% bovine serum albumin, in PBST for 30 minutes at 37°C.
- viii) Rinse four times with PBST, 5 minutes for each rinse. The slides or plastic culture plates can be gently agitated during the rinses.
- ix) Prepare a solution of purified antibody or serum to SVCV in PBST, at the appropriate dilution (which has been established previously or as given by the reagent supplier).
- x) Incubate the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur.
- xi) Rinse four times with PBST.
- xii) Incubate the cell monolayers with a solution of fluorescein isothiocyanate (FITC)-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These FITC antibodies are most often rabbit or goat antibodies.
- xiii) Rinse four times with PBST.
- xiv) View the treated cell monolayers on plastic substrates immediately, or mount the slides or cover-slips using glycerol saline at pH 8.5, or a commercially available mountant.
- xv) Examine under incident ultraviolet (UV) light using a microscope with ×10 eye pieces and ×20 or ×40 objective lenses having numerical apertures of >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

4.10. Other serological methods

Not applicable

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The method for surveillance of apparently healthy populations susceptible fish populations for declaration of freedom from infection with SVCV is inoculation of cell culture with tissue homogenates ~~extracts~~ (as described in Section 4.3–4.5) ~~to demonstrate absence of the virus.~~

6. Corroborative diagnostic criteria

This Section only addresses the diagnostic test results for detection of infection in the ~~presence~~ absence (Section 6.1.) or in the presence ~~absence~~ of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status¹

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link ~~(s)~~ to an infected population. Geographical proximity to, or movement of animals or animal products or equipment, ~~etc.~~, from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with SVCV shall be suspected if: ~~a positive result has been obtained on at least one animal from~~ at least one of the following ~~diagnostic tests~~ criteria is met:

- i) Positive result by conventional RT-PCR ~~a recommended molecular or antigen or antibody detection test~~
- ii) SVCV-typical CPE. Cytopathic effect in cell culture ~~(viruses)~~

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with SVCV is considered to shall be confirmed if, in addition to the criteria in Section 6.1.1. ~~positive results has been obtained on at least one animal from two test used in the following combination~~ the following criterion is met:

- i) Pathogen isolation ~~AND Conventional~~ SVCV-typical CPE in cell culture followed by virus identification by conventional RT-PCR test followed by ~~and amplicon sequencing.~~

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.2. Clinically affected animals

Clinical signs are not pathognomonic for infection with SVCV ~~a single disease~~; however they may narrow the range of possible diagnoses. ~~[For many diseases, especially those affecting mollusc, 'clinical signs' are extremely limited and mortality may be the only or most dominant observation.]~~

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with SVCV shall be suspected if at least one of the following criteria is ~~are~~ met:

¹ For example transboundary *commodities*.

Annex 11 (contd)

- i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality
- ii) Positive result by conventional RT-PCR ~~a recommended molecular or antigen or antibody detection test on at least one animal~~
- iii) Positives result by antigen ELISA or IFAT or immunohistochemistry
- iv) Positive result by IFAT
- v) Positive result by immunohistochemistry
- vi) SVCV-typic CPE ~~Cytopathic effect~~ in cell culture.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with SVCV is considered to shall be confirmed if, in addition to the criteria in Section 6.2.1., ~~positive results has been obtained on at least one animal from two test used in the following combination~~ the following criterion is met:

- i) Pathogen isolation ~~AND~~ Conventional SVCV-typic CPE in cell culture followed by virus identification by conventional RT-PCR test followed by and amplicon sequencing.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with SVCV are provided in Table 6.3. (note: no data are currently available). This information can be used for the design of surveys for infection with SVCV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to atleast level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

Table 6.1. Diagnostic performance of tests recommended for surveillance or diagnosis

Test type	Test purpose	Source population	Tissue/ sample type	Species	DSe (n)	DSp (n)	Reference test	Citation
Cell culture	Surveillance, diagnosis	≡	Tissue homogenates	≡	Not yet available	Not yet available	≡	≡
RT-PCR	Surveillance, diagnosis	≡	Tissue homogenates	≡	Not yet available	Not yet available	≡	≡
RT-PCR	Surveillance, diagnosis	≡	Cell culture	≡	Not yet available	Not yet available	≡	≡
RT-LAMP*	Surveillance	Live imported fish	Spleen, kidney and brain homogenate	Common carp, koi, goldfish	92.6 (27)	98.2 (445)	Virus isolation	Liu et al., 2008

DSe = diagnostic sensitivity; DSp = diagnostic specificity; n = number of samples used in the study;
RT-LAMP: = real-time loop mediated isothermal amplification. *Listed as suitable test

7. References

- AHNE W. (1976). Untersuchungen über die Stabilität des karpfenpathogenen Virusstammes 10/3. *Fisch und Umwelt*, **2**, 121–127.
- AHNE W. (1982). Vergleichende Untersuchungen über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV). *Zbl. Vet. Med. B*, **29**, 457–476.
- AHNE W. (1986). Unterschiedliche biologische Eigenschaften 4 cyprinidenpathogener Rhabdovirusisolate. *J. Vet. Med. B*, **33**, 253–259.
- AHNE W., BJORKLUND H.V., ESSBAUER S., FIJAN N., KURATH G. & WINTON J.R. (2002). Spring viremia of carp (SVC). *Dis. aquat. Org.*, **52**, 261–272.
- AHNE W. & HELD C. (1980). Untersuchungen über die viruzide Wirkung von Actomar® K₃₀ auf fischpathogene Viren. *Tierärztl. Umsch.*, **35**, 308–318.
- ALIKIN Y.S., SHCHELKUNOV I.S., SHCHELKUNOVA T.I., KUPINSKAYA O.A., MASYCHEVA V.I., KLIMENKO V.P. & FADINA V.A. (1996). Prophylactic treatment of viral diseases in fish using native RNA linked to soluble and corpuscular carriers. *J. Fish Biol.*, **49**, 195–205.
- BEKESI L. & CSONTOS L. (1985). Isolation of spring viraemia of carp virus from asymptomatic broodstock carp, *Cyprinus carpio* L. *J. Fish Dis.*, **8**, 471–472.
- BILLARD R. & BERNI P. (2004). Trends in cyprinid polyculture. *Cybium*, **28**, 255–261.**
- BJORKLUND H.V., HIGMAN K.H. & KURATH G. (1996). The glycoprotein genes and gene junctions of the fish rhabdoviruses spring viremia of carp virus and hirame rhabdovirus – analysis of relationships with other rhabdoviruses. *Virus Res.*, **42**, 65–80.
- DE KINKELIN P. & LE BERRE M. (1974). Rhabdovirus des poissons II. Propriétés *in vitro* du virus de la virémie printanière de la carpe. *Ann. Microbiol. (Inst. Pasteur)*, **125 A**, 113–124.
- DIKKEBOOM A.L., RADI C., TOOHEY-KURTH K., MARCQUENSKI S., ENGEL M., GOODWIN A.E., WAY K., STONE D.M. & LONGSHAW C. (2004). First Report of Spring Viremia of Carp Virus (SVCV) in Wild Common Carp in North America. *J. Aquat. Anim. Health*, **16**, 169–178.
- DIXON P.F. (2008). Virus diseases of cyprinids. *In: Fish Diseases*, Vol. 1. Eiras J.C., Segner H., Wahli, T. & Kapoor, B.G. eds. Science Publishers, Enfield, New Hampshire, USA, 87–184.
- DIXON P.F. & LONGSHAW C.B. (2005). Assessment of commercial test kits for identification of spring viraemia of carp virus. *Dis. Aquat. Org.*, **67**, 25–29.
- EMMENEGGER E.J. & KURATH G. (2008). DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus. *Vaccine*, **26**, 6415–6421.
- FIJAN N. (1988). Vaccination against spring viraemia of carp. *In: Fish Vaccination*, Ellis A.E., ed. Academic Press, London, UK, 204–215.
- FIJAN N. (1999) Spring viraemia of carp and other viral diseases and agents of warm-water fish. *In: Fish Diseases and Disorders Vol. 3: Viral, Bacterial, and Fungal Infections*, Woo P.T.K. & Bruno D.W., eds. CAB International, London, UK, pp. 177–244.
- GARVER K.A., DWILOW A.G., RICHARD J., BOOTH T.F., BENIAC D.R. & SOUTER B.W. (2007). First detection and confirmation of spring viraemia of carp virus in common carp, *Cyprinus carpio* L., from Hamilton Harbour, Lake Ontario, Canada. *J. Fish Dis.*, **30**, 665–671.
- HOFFMANN B., SCHÜTZE H. & METTENLEITER T.C. (2002). Determination of the complete genomic sequence and analysis of the gene products of the virus of Spring Viremia of Carp, a fish rhabdovirus. *Virus Res.*, **84**, 89–100.
- KIRPICHNIKOV V.S., ILYASOV I., SHART L.A., VIKHMAN A.A., GANCHENKO M.V., OSTASHEVSKY A.L., SIMONOV V.M., TIKHONOV G.F. & TJURIN V.V. (1993). Selection of Krasnodar common carp (*Cyprinus carpio* L.) for resistance to dropsy: principal results and prospect. *In: Genetics in Aquaculture*, Proceedings of the Fourth International Symposium on Genetics in Aquaculture, Graham A.E. & Gall G.A.E., eds. Elsevier, Amsterdam, Netherlands, 7–20.

Annex 11 (contd)

KIRYU I., SAKAI T., KURITA J. & IIDA T. (2007). Virucidal effect of disinfectants on spring viremia of carp virus. *Fish Pathol.*, **42**, 111–113.

KOUTNA M., VESELY T., PSIKAL I. & HULOVÁ J. (2003). Identification of spring viraemia of carp virus (SVCV) by combined RT-PCR and nested PCR. *Dis. Aquat. Org.*, **55**, 229–235.

LIU H., GAO L., SHI X., GU T., JIANG Y. & CHEN H. (2004). Isolation of spring viraemia of carp virus (SVCV) from cultured koi (*Cyprinus carpio koi*) and common carp (*C. carpio carpio*) in PR China. *Bull. Eur. Assoc. Fish Pathol.*, **24**, 194–202.

LIU Z., TENG Y., XIE X., LI H., LV J., GAO L., TIAN F., JIANG Y., CHU Z., XIE C. & LIU H. (2008). Development and evaluation of a one-step loop-mediated isothermal amplification for detection of spring viraemia of carp virus. *J. Appl. Microbiol.*, **105**, 1220–1226.

MISK E., GARVER K., NAGY E., ISAAC S., TUBBS L., HUBER P., AL-HUSSINEE L. & LUMSDEN J.S. (2016). Pathogenesis of spring viremia of carp virus in emerald shiner *Notropis atherinoides* Rafinesque, fathead minnow *Pimephales promelas* Rafinesque and white sucker *Catostomus commersonii* (Lacepede). *J. Fish Dis.*, **39**, 729–739.

ORESHKOVA S.F., SHCHELKUNOV I.S., TIKUNOVA N. V., SHCHELKUNOVA T.I., PUZYREV A.T. & ILYICHEV A.A. (1999). Detection of spring viremia of carp virus isolates by hybridization with non-radioactive probes and amplification by polymerase chain reaction. *Virus Res.*, **63**, 3–10.

PETERS F. & NEUKIRCH M. (1986) Transmission of some fish pathogenic viruses by the heron, *Ardea cinerea*. *J. Fish Dis.*, **9**, 539–544.

RUANE N.M., RODGER H.D., MCCARTHY L.J., SWORDS D., DODGE M., KERR R.C., HENSHILWOOD K. & STONE D.M. (2014). Genetic diversity and associated pathology of rhabdovirus infections in farmed and wild perch *Perca fluviatilis* in Ireland. *Dis. Aquat. Org.*, **112**, 121–130.

SHEPPARD A.M., LEDEUFF R.-M., MARTIN P.D., WOOLFORD G., WAY K. & STONE D.M. (2007). Genotyping spring viraemia of carp virus and other piscine vesiculo-like viruses using reverse hybridisation. *Dis. Aquat. Org.*, **76**, 163–168.

SHIMAHARA Y., KURITA J., NISHIOKA T., KIRYU I., YUASA K., SAKAI T., OSEKO N., SANO M. & DIXON P. (2016). Development of an improved RT-PCR for specific detection of spring viraemia of carp virus. *J. Fish Dis.*, **39**, 269–275.

SHIVAPPA R.B., SAVAN R., KONO T., SAKAI M., EMMENEGGER E., KURATH G. & LEVINE J F (2008). Detection of spring viraemia of carp virus (SVCV) by loop-mediated isothermal amplification (LAMP) in koi carp, *Cyprinus carpio* L. *J. Fish Dis.*, **31**, 249–258.

SIWICKI A.K., POZET F., MORAND M., KAZUŃ B., TRAPKOWSKA S. & MAŁACZEWSKA J. (2002). Influence of methisoprinol on the replication of rhabdoviruses isolated from carp (*Cyprinus carpio*) and catfish (*Ictalurus melas*): *in vitro* study. *Polish J. Vet. Sci.*, **6**, 47–50.

STONE D.M., AHNE W., DENHAM K.D., DIXON P.F., LIU C.T.Y., SHEPPARD A.M., TAYLOR G.R. & WAY K. (2003). Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. *Dis. Aquat. Org.*, **53**, 203–210.

SVETLANA J., DOBRILA J.-D. & RADOSAVLJEVI V. (2004). Dissemination of spring viraemia of carp (svc) in serbia during the period 1992–2002. *Acta Veterinaria* (Beograd), **54**, 289–299.

TENG Y., LIU H., LV J.Q., FAN W.H., ZHANG Q.Y. & QIN Q.W. (2007). Characterization of complete genome sequence of the spring viremia of carp virus isolated from common carp (*Cyprinus carpio*) in China. *Arch. Virol.*, **152**, 1457–1465.

YUE Z., TENG Y., LIANG C., XIE X., XU B., ZHU L., LEI Z., HE J., LIU Z., JIANG Y., LIU H. & QIN Q. (2008). Development of a sensitive and quantitative assay for spring viremia of carp virus based on real-time PCR. *J. Virol. Methods*, **152**, 43–48.

UNOFFICIAL VERSION

Annex 11 (contd)

ZHANG N.Z., ZHANG L.F., JIANG Y.N., ZHANG T. & XIA C. (2009). Molecular analysis of spring viraemia of carp virus in China: A fatal aquatic viral disease that might spread in East Asian. *PLoS ONE*, **4**, pp 1–9.

*

* *

NB: There are OIE Reference Laboratories for Spring viraemia of carp
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratories for any further information on
Spring viraemia of carp

NB: First adopted in 1995 as spring viraemia of carp. Most recent updates adopted in 2012.

CHAPTER 2.1.3.

INFECTION WITH BATRACHOCHYTRIUM SALAMANDRIVORANS

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter. One comment is inserted in the text below.

1. Scope

Infection with *Batrachochytrium salamandrivorans* (Bsal) means infection of amphibians with the pathogenic agent *Batrachochytrium salamandrivorans*, of the Division Chytridiomycota and Order Rhizophydiales ~~Genus *Batrachochytrium* and Family Incertae sedis.~~

2. Disease information**2.1. Agent factors****2.1.1. Aetiological agent**

The type strain of the pathogenic chytrid fungal agent *Batrachochytrium salamandrivorans* (Bsal) type strain is AMFP13/1. Three more isolates have been described (Martel *et al.*, 2014) but no information is available on genetic structuring or phenotypic variation. Phylogenetic analyses show that Bsal forms a clade with its sister species *B. dendrobatidis* (Martel *et al.*, 2013). The genome size of the type strain was determined at 32.6 Mb with 10,138 protein-coding genes predicted (Farrer *et al.*, 2017). The contribution of these proteins to virulence is currently not clear.

2.1.2. Survival and stability inside the host tissues ~~in processed or stored samples~~

Bsal is an intracellular pathogen that develops inside epidermal cells. The presence of Bsal could be demonstrated using real-time polymerase chain reaction (qPCR) on dorsal skin swabs up to 7 days on average post-mortem and using histopathology of dorsal skin tissue up to 3 days on average post-mortem (Thomas *et al.*, 2018). It is not clear how long Bsal can survive inside tissues of a dead host and how long a dead host remains infectious. ~~Storage of tissues or skin swabs in 70% ethanol or at 20°C allows detection of Bsal using qPCR for more than 150 years as demonstrated by analysis of museum specimens (Martel *et al.*, 2014).~~

2.1.3. Survival and stability outside the host

Encysted spores have been shown to remain infectious in pond water for up to at least 31 days (Stegen *et al.*, 2017) and are considered more environmentally-resistant in the environment compared with zoospores. Experimentally inoculated forest soil was demonstrated to remain infectious to fire salamanders for 48 hours (Stegen *et al.*, 2017). However, Bsal DNA was detected up to 28 weeks in contaminated forest soil for up to 28 weeks (Stegen *et al.*, 2017). ~~However, Whether~~ this reflects the presence of viable Bsal organisms is not clear. The effect of desiccation desiccation on Bsal survival has not been studied.

For inactivation methods, see Section 2.4.5.

2.2. Host factors**2.2.1. Susceptible host species**

Species that fulfil the criteria for listing as susceptible to infection with Bsal according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: alpine newt (*Ichthyosaura alpestris*), blue-tailed fire-bellied newt (*Cynops cyanurus*), fire salamander (*Salamandra salamandra*), eastern newt

(*Nothophthalmus viridescens*), French cave salamander (*Hydromantes strinatii*), Italian newt (*Lissotriton italicus*), yellow spotted newt (*Neurergus crocatus*), Japanese fire-bellied newt (*Cynops pyrrhogaster*), northern spectacle salamander (*Salamandrina perspicillata*), Tam Dao salamander (*Paramesotriton deloustali*), rough-skinned newt (*Taricha granulosa*), sardinian brook salamander (*Euproctus platycephalus*) and Spanish ribbed newt (*Pleurodeles waltli*) (under study).

Family	Scientific name	Common name
Plethodontidae	<i>Hydromantes strinatii</i>	French cave salamander
Salamandridae	<i>Cynops cyanurus</i>	blue-tailed fire-bellied newt
	<i>Cynops pyrrhogaster</i>	Japanese fire-bellied newt
	<i>Euproctus platycephalus</i>	sardinian brook salamander
	<i>Ichthyosaura alpestris</i>	Alpine newt
	<i>Lissotriton italicus</i>	Italian newt
	<i>Neurergus crocatus</i>	yellow spotted newt
	<i>Nothophthalmus viridescens</i>	eastern newt
	<i>Paramesotriton deloustali</i>	Tam Dao salamander
	<i>Pleurodeles waltli</i>	Spanish ribbed newt
	<i>Salamandrina perspicillata</i>	northern spectacle salamander
	<i>Salamandra salamandra</i>	fire salamander
	<i>Taricha granulosa</i>	rough-skinned newt

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* are: [under study]

2.2.3. Non-susceptible species

Species that have been found non-susceptible to infection with Bsal according to Chapter 1.5. of the *Aquatic Code* are: [under study]

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

Bsal is a pathogenic agent that mainly affects urodeles. Evidence from experimental infections and disease outbreaks in the wild and in captivity show that at least most, if not all, species of the family Salamandridae, as well as species of the family Hynobiidae are likely to become infected when exposed to Bsal. However, differences in susceptibility to infection between species do exist: for example, for fire salamanders (*Salamandra salamandra*), the infectious dose of Bsal was determined to be a theoretical one zoospore, whereas a significantly higher dose was necessary to infect Alpine newts (*Ichthyosaura alpestris*); Stegen *et al.*, 2017) and one western Palearctic species (*Lissotriton helveticus*) may be more resistant to infection (Martel *et al.*, 2014). For the largest family of salamanders (Plethodontidae), little information is currently available; at least one European species (*Speleomantes strinatii*) can be infected but other, North American species (*Gyrinophilus porphyriticus*, *Plethodon glutinosus*, Ambystomatidae) seem less susceptible to infection (Martel *et al.*, 2014). Susceptibility of the family of *Cryptobranchidae* is not clear, with a single infection found in a farmed Chinese giant salamander (*Andrias davidianus*; Zhiyong *et al.*, 2018). No information is available on the urodele families *Proteidae*, *Rhyacotritonidae* and *Amphiumidae*. Bsal infection in anurans has only been detected in two species, in captivity, the wild and in lab trials (Nguyen *et al.*, 2017; Stegen *et al.*, 2017).

Thus far, infections with Bsal have been demonstrated only in amphibians post-metamorphosis. In one experimental infection trial, larvae of fire salamanders were exposed to Bsal, but did not become were not infected (Van Rooij *et al.*, 2015). The extent to which factors such as like-age and sex affect susceptibility to infection post-metamorphosis is unknown.

In Europe, Bsal has been detected in captive collections of urodeles (Fitzpatrick *et al.*, 2018, Sabino-Pinto *et al.*, 2015) and the pet trade in salamanders and newts has been hypothesised to play a central role in the distribution of this fungus (Fitzpatrick *et al.*, 2018; Yap *et al.*, 2015; Zhiyong *et al.*, 2018). Hence, urodeles that come into contact with traded urodeles, either directly (by-via co-housing or contact of-with wild animals with-or released or captive animals) or indirectly (via materials, contaminated water or soil) come in contact with traded urodeles, may have a high likelihood of exposure to infection with be more likely to contract Bsal-infection.

2.2.5. Distribution of the pathogen in the host

Bsal only infects the skin, where it remains limited to the epidermis.

2.2.6. Aquatic animal reservoirs of Persistent-infection

A large number of salamanders, mainly belonging to the families Salamandridae and Hynobiidae, may survive episodes of infection (for example Alpine newts) or be considered tolerant, resulting in persistent subclinical infections. Although persistent infection has not been demonstrated for all species, in the native Bsal range in east Asia, Bsal infection and disease dynamics appear to be consistent for all species examined and appear capable of long-term persistent infections (Laking *et al.*, 2017; Martel *et al.*, 2014; Zhiyong *et al.*, 2018).

In its invasive range, persistent infections (e.g. in Alpine newts) have been implicated in the **extirpation local extinction** of a highly susceptible species (fire salamanders). It is currently not clear which of the species, mentioned in Section 2.2.1 may sustain persistent infections in the invasive Bsal range. At least some species (the best-known example is the fire salamander) are highly susceptible and invariably die shortly ~~briefly~~ after exposure (Martel *et al.*, 2014; Stegen *et al.*, 2017), making which ~~would make~~ them unlikely to sustain persistent infections.

It is not known whether other, biotic reservoirs of Bsal exist.

2.2.7. Vectors

There is evidence that birds may carry zoospores attached to their ~~the~~ feet of birds (Stegen *et al.*, 2017), which may and thus may act as vectors for Bsal.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

In its native range in east Asia, Bsal has been demonstrated to be present in the wild at a prevalence of between 2 and 4% on average (data from China [People's Rep. of], Japan, Thailand, and Vietnam; Laking *et al.*, 2017; Martel *et al.*, 2014; Zhiyong *et al.*, 2018), but in the absence of any observed morbidity or mortality under natural conditions. In some populations (*Paramesotriton hongkongensis*), prevalence may reach 50% (Zhiyong *et al.*, 2018). In its invasive range in Europe, Bsal was present in a population of fire salamanders at a prevalence of between 25 and 63% (Stegen *et al.*, 2017). **In captive collections of urodeles in Europe, Bsal occurrence and associated mortality has been were** detected in **captive collections of urodeles in Europe, including** Germany (1), the United Kingdom (4), Belgium (1), the Netherlands (2) and Spain (1) (number in brackets indicates number of collections). When left untreated, morbidity and mortality can reach 100%, at least in members of the genus *Salamandra*.

Morbidity, mortality and minimum infectious dose vary considerably between species (Martel *et al.*, 2014; Stegen *et al.*, 2017). Based on natural outbreaks in captivity and in the wild and in ~~on~~ infection trials, the case morbidity and case mortality rate in fire salamanders can reach 100%, independent of the initial level of Bsal exposure. This has resulted in the loss of over 99.9% of the fire salamander population at the Bsal index outbreak site in the Netherlands (Spitzen-van der Sluijs *et al.*, 2016). All tested western Palearctic urodeles, except for *Lissotriton helveticus* and *Salamandrella keyserlingii*, showed 100% morbidity and mortality when exposed to a single, high dose of Bsal (Martel *et al.*, 2014). However, at least for Alpine newts, the case morbidity and case fatality rates depend on the Bsal dose that the animal is exposed to: a high dose resulting in the highest mortality, while a low dose does not necessarily result in morbidity or mortality.

~~It is important to mention that~~ Morbidity and mortality also depend on environmental temperature. For the Bsal type strain, temperatures above 20°C reduces the level of ~~tempers~~ infection and temperatures above 25°C eventually result in killing of Bsal and elimination of infection (Bloom *et al.*, 2015b-2015a). Exposure of infected animals to conditions that inhibit Bsal growth may thus result in non-clinical or sub-clinical infections in susceptible species.

2.3.2. Clinical signs, including behavioural changes

Chytridiomycosis caused by Bsal may be accompanied by a combination of the following signs: epidermal ulcerations (ranging from discrete ~~tiny~~ to extensive), excessive skin shedding, skin haemorrhages and/or fluid loss, anorexia, apathy, abnormal body postures and convulsions and death (Martel *et al.*, 2013).

2.3.3. Gross pathology

Skin anomalies (haemorrhages, ulcerations, presence of sloughed skin) are the main pathological findings (Martel *et al.*, 2013).

2.3.4. Modes of transmission and life cycle

Colonial or monocentric thalli of this fungus develop inside host epidermal cells and produce motile zoospores or walled, encysted spores, both of which are infectious stages. Zoospores are released through one or several discharge tubes. While motile spores actively swim towards a suitable substrate (e.g. a host), the encysted spores float at the water-air interface and passively adhere to a passing host (Stegen *et al.*, 2017). *In vitro*, developing thalli form fine rhizoids. Mature thalli *in vitro* are between 16 and 50 µm in diameter, *in vivo* between 7 and 17 µm; zoospores are approximately 5 µm in diameter. Motile zoospores are roughly spherical, the nucleus is located outside of the ribosomal mass, with aggregated ribosomes, multiple mitochondria and numerous lipid globules. The position of the

non-flagellated centriole in free swimming zoospores varies from angled to parallel to the kinetosome (Martel *et al.*, 2013).

There are no indications of vertical transmission. However, this cannot be excluded in species giving birth to metamorphosed offspring (e.g. *Salamandra atra*, *Salamandra lanzai*, *Lyciasalamandra helverseni*). Horizontal transmission occurs through direct contact or contact with contaminated soil or water (Stegen *et al.*, 2017). Infectious stages include the motile zoospore and the environmentally resistant encysted spores (Stegen *et al.*, 2017). Infections can be reproduced under experimental conditions by topically applying a Bsal inoculum on the dorsum of amphibians and housing the exposed animals at 15°C (Martel *et al.*, 2013; 2014; Stegen *et al.*, 2017). This inoculum can either contain motile zoospores or the immobile, encysted spores.

Pathways of Bsal dispersal within Europe are poorly understood but may be anthropogenic (e.g. through contaminated material). Zoospores attach to bird feet, suggesting birds may spread Bsal over larger distances (Stegen *et al.*, 2017). Direct animal-to-animal contact is necessary for transmission of Bsal: salamanders only separated by 1 cm from infected conspecifics were not infected in laboratory trials, in contrast to co-housed animals (Spitzen-van der Sluijs *et al.*, 2018). Overall, dispersal ability of Bsal in Europe currently seems limited: Bsal was found not to be transmitted to a neighbouring site in the Netherlands, despite being downstream of a small stream, and the current distribution of Bsal in Europe is probably not continuous (Spitzen-van der Sluijs *et al.*, 2018).

Although Bsal dispersal between populations is now hypothesised to be mainly human mediated, other factors (e.g. wildlife, water) may play key roles and critical knowledge about Bsal dispersal is currently lacking.

2.3.5. Environmental and management factors

The Bsal type strain AMFP13/1 tolerates temperatures up to 25°C but is killed at higher temperatures (Bloo *et al.*, ~~2015b–2015a~~). As Bsal infections have been demonstrated in aquatic newts at water temperatures above 25°C (Laking *et al.*, 2017; Zhiyong *et al.*, 2018), it is likely, however, that thermal tolerance may be Bsal lineage dependent. A temperature of 4°C results in slower progression build-up of infection but does not reduce morbidity or mortality (Stegen *et al.*, 2017). Desiccation is likely to be poorly tolerated by Bsal, although data are currently lacking, and the encysted spore may be resistant to drying (Stegen *et al.*, 2017; Van Rooij *et al.*, 2015). It is not known to what extent Bsal tolerates freezing.

Co-occurrence of highly susceptible species such as fire salamanders with less susceptible species, such as Alpine newts may facilitate density independent disease dynamics that lead to the extirpation local extinction of the highly susceptible species (Stegen *et al.*, 2017).

Barriers to pathogen dispersal, for example those preventing migration of infected hosts such as amphibian fences or roads, or those preventing transmission by potential Bsal vectors including humans, fomites and wildlife, may prevent transmission at small spatial scales (Spitzen-van der Sluijs *et al.*, 2018).

2.3.6. Geographical distribution

Asia is currently considered the region of origin of Bsal (Martel *et al.*, 2014), where the infection appears to be endemic in amphibian communities across a wide taxonomic, geographical and environmental range, albeit at a low prevalence between 2 and 4% (Zhiyong *et al.*, 2018). In Asia, Bsal was shown to be widely present in urodele populations in China (People's Rep. of), Japan, Thailand and Vietnam. East Asia is presumed to be the native range of the fungus (Laking *et al.*, 2017; Martel *et al.*, 2014; Zhiyong *et al.*, 2018).

Europe is considered the invasive range of the fungus where Bsal was first identified during a mortality event in fire salamanders (*Salamandra salamandra*) in Bunderbos, the Netherlands (Martel *et al.*, 2013). In Europe, Bsal was detected by surveys of wild susceptible species in Belgium, Germany and the Netherlands (Martel *et al.*, 2014; Spitzen-van der Sluijs *et al.*, 2016), and in captive urodele populations in Belgium, Germany, the Netherlands, Spain, and the United Kingdom (Fitzpatrick *et al.*, 2018; Sabino-Pinto *et al.*, 2015).

Bsal has not been reported in Africa or the Americas.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Not available.

2.4.2. Chemotherapy including blocking agents

A combined treatment using Polymyxin E, voriconazole and a temperature regime of 20°C has been shown to be effective in eradicating Bsal from infected hosts (Bloo *et al.*, ~~2015c–2015b~~). If the treatment is not performed properly and does not achieve eradication, low level carriers are created and the likelihood of Bsal detection, is reduced.

2.4.3. Immunostimulation

Not available.

UNOFFICIAL VERSION

2.4.4. Breeding resistant strains

~~Breeding resistant strains is one of the few options for long term sustainable disease mitigation.~~
No information available.

2.4.5. Inactivation methods

Bsal is sensitive to a wide variety of disinfectants (Van Rooij *et al.*, 2015). Inactivation using formalin has been shown to hamper DNA detection using real-time PCR ~~qPCR~~. Bsal is killed within 30 seconds in 70% ethanol (Van Rooij *et al.*, 2017). Inactivation in 70% ethanol allows for subsequent molecular tests yet is less suitable for histopathology. The Bsal type strain AMFP 13/1 is killed at temperatures exceeding 25°C; consequently, inactivation of this fungus can be achieved through heat treatment by autoclaving (Martel *et al.*, 2013).

2.4.6. Disinfection of eggs and larvae

No information available.

2.4.7. General husbandry

In captivity, pathogen detection is difficult due to low prevalence in subclinically infected animals that often carry Bsal at low intensities (Martel *et al.*, 2014; Zhiyong *et al.*, 2018). These subclinically infected animals often belong to (but are not restricted to) taxa of Asian urodeles. Highly susceptible species (such as fire salamanders—Salamandra salamandra) may serve a sentinel function. Temperature regimes in captivity may strongly interfere with pathogen detection. Temperatures higher than 20°C (and below 25°C) severely impair pathogen proliferation in the host skin (Bloo *et al.*, 2015b-2015a) and may result in infections that cannot be detected.

Heat treatment can be used to clear infection with Bsal in thermotolerant salamander species (Bloo *et al.*, 2015a).

3. Specimen selection, sample collection, transportation and handling

This Section draws on information from Sections 2.2., 2.3. and 2.4. to identify populations, individuals and samples which are most likely to be infected.

3.1. Selection of populations and individual specimens

In case of disease or mortality in urodeles in captivity, sampling should be focused primarily on diseased or moribund animals (i.e. those showing skin lesions and abnormal behaviour). In a population with ongoing disease and mortality, live but diseased animals are preferentially sampled. The second choice is dead animals. Only freshly dead animals should be sampled as detectability of Bsal deteriorates post-mortem (Thomas *et al.*, 2018). However, in the absence of diseased or freshly dead animals, apparently healthy animals can be sampled.

Similarly, in wild populations, samples should be taken preferentially from diseased or moribund or freshly dead animals ~~should preferentially be sampled, but; however,~~ as these may quickly be removed (i.e. through predation, scavenging) only healthy animals may ~~only~~ be available. Populations which have declined or where dead animals have been observed should be targeted.

3.2. Selection of organs or tissues

The only relevant tissue is skin tissue and probably only from amphibians post-metamorphosis. Both invasive (skin biopsies) and non-invasive (cotton tipped swabs) samples ~~sampling~~ are appropriate, given the apical shedding of Bsal spores. In dead animals, dorsal skin is the preferred tissue, given its slower post-mortem decay (Thomas *et al.*, 2018).

3.3. Samples or tissues not suitable for pathogen detection

Any other t[issues] other than skin is are not suitable for the detection of Bsal in amphibians.

3.4. Non-lethal sampling

Non-lethal sampling is possible, either by collecting skin biopsies (toeclips or tailclips) or by non-invasively collecting samples using cotton tipped swabs. The latter is preferred given its minimal impact on animal welfare well-being. As Bsal is limited to the superficial skin layers of the amphibian host, non-lethal sampling results are equivalent to lethal sampling results. ~~In the absence of other, Bsal-specific diagnostic tests (other than the laborious isolation of the fungus),~~ Large numbers of animals can be sampled using skin swabs with minimal effects on animal welfare. Cotton tipped swabs should be rubbed firmly over the abdomen (10 times), the underside of a foot (10 times) and the ventral tail (10 times) using the tip of the swab. The use of disposable gloves for manipulating amphibians is highly recommended.

Annex 12 (contd)

3.5. Preservation of samples for submission

3.5.1. Samples for pathogen isolation

Bsal isolation is a ~~very~~ laborious procedure, requiring up to two months ~~to obtain for obtaining~~ a pure culture from a clinical sample. Isolation from animals that died due to Bsal infection is hampered by bacterial overgrowth. The best sample for Bsal isolation is a diseased, living animal, which is euthanised just prior to an isolation attempt. Before sampling diseased animals should be kept at temperatures between 5 and 15°C to avoid clearance of infection (Bloom *et al.*, ~~2015b-2015a~~).

3.5.2. Preservation of Fixed samples for molecular detection

Tissue samples for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (undenatured) ethanol. The recommended ratio of ethanol to tissue is 10:1. The use of lower grade (laboratory or industrial grade) ethanol is not recommended. If material cannot be fixed it may be frozen.

Skin swabs should be stored dry and preferably frozen.

3.5.3. Fixed Preservation of samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Skin samples for histopathology should be fixed immediately after collection. The recommended ratio of formalin (10%) to tissue is 10:1.

3.5.4. Fixed Preservation of samples for electron microscopy

For transmission electron microscopy, skin samples can be fixed in glutaraldehyde in 0.05 M sodium cacodylate buffer and 1% osmium tetroxide post-fixation (Martel *et al.*, 2013).

3.5.5. Samples for other tests

Not applicable.

3.6. Pooling of samples

Pooling of up to ~~four~~ five skin swab samples appears to allow reliable detection of Bsal in clinically affected animals (Sabino-Pinto *et al.*, ~~2018-2019a; 2019b~~) but estimates ~~of~~ on the impact on diagnostic performance of the test characteristics have not been determined. Given low infection intensities in subclinically infected animals, sampling and testing of individual animals is recommended.

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

- +++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;
- ++ = Suitable method(s) but may need further validation;
- + = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;

Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of **apparently** healthy animals and investigation of clinically affected animals

Method [amend or delete as relevant]	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts	+	+	+	1	+	+	+	1				1
Histopathology ³	+	+	+	1	++	++	++	1				1
Cell or artificial media culture									+	+	+	1
Real-time PCR	+++	+++	+++	2	+++	+++	+++	2	+++	+++	+++	2
Conventional PCR												
Amplicon sequencing ⁴												
<i>In-situ</i> hybridisation												
LAMP												
Lateral flow assay	+	+	+	1	+	+	+	1				1
Immunohistochemistry												

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2.); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification.

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Early and juvenile life stages have been defined in Section 2.2.4.

³Cytopathology and histopathology can be validated if the results from different operators has been statistically compared. ⁴Sequencing of the PCR product.

Shading indicates the test is inappropriate or should not be used for this purpose.

EU comment

We have identified what it seems to be an apparent contradiction in pages 90 to 92, table 4.1 and the text in section 4.4.1.

In Section 4, where the keys are described for the diagnostic methods, key +++ corresponds to recommended methods validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway

According to section 4.4.1, TaqMan PCR has been partially validated to level 2 without however, stating its intended purpose. Furthermore, SYBR green real-time PCR also needs further validation.

Based on the above, we believe that real-time PCR in table 4.1 should therefore be scored as ++ (not as +++).

UNOFFICIAL VERSION

4.1. Wet mounts

Wet mounts of skin scraping or pieces of shed skin can be examined at magnification 10× using light microscopy. The presence of motile spores of approximately 5 µm are indicative of amphibian chytrid infection.

4.2. Histo- and cytopathology

~~No reports are available on the use of cytology.~~ Histopathology of skin in amphibian post-metamorphosis may provide strong indications of Bsal infection. In a haematoxylin/eosin ~~staining of skin~~ stained sections, histopathological evidence suggestive of Bsal infections of skin, is multifocal epidermal necrosis with loss of distinction between layers of keratinocytes associated with myriad intracellular and extracellular chytrid-type fungal thalli provides histopathological evidence of Bsal infection (Martel *et al.*, 2013; White *et al.*, 2016). Using immunohistochemistry, Bsal thalli can be stained, which aids in detecting low level infections (Thomas *et al.*, 2018). Histopathology is highly indicative, yet does not allow ~~specific definitive~~ identification of Bsal, which needs further confirmation. In randomly collected skin samples from experimentally infected salamanders, histopathology was capable of detecting Bsal in only a minority of the samples (Thomas *et al.*, 2018). In dead animals, post-mortem decay of the epidermis may mask the lesions (Thomas *et al.*, 2018). Lesions can be so extensive, that the epidermis is entirely eroded and no fungal thalli can be observed. Mild infections can be missed due to the multifocal and small lesions (Thomas *et al.*, 2018). ~~For asymptotically~~ In subclinically infected animals, sensitivity should be rated low. ~~Sensitivity.~~ In clinically affected animals, sensitivity and specificity of histopathology and immunohistochemistry have not been quantified.

No reports are available on the use of cytopathology.

4.3. Cell or artificial media culture for isolation

Bsal can be isolated and cultured on artificial media, yet this is a laborious and difficult procedure, typically requiring between 4 weeks and 2 months. There is a significant probability of bacterial overgrowth, which hampers fungal isolation, resulting in poor sensitivity. The protocol of Fisher *et al.* (2018) can be used. Small (approximately 1 mm²) pieces of skin from an infected, diseased animal should first be thoroughly cleaned by wiping through agar plates. The cleaned pieces of skin can then each be transferred to a well of a 96-well plate, containing tryptone-gelatin hydrolysate lactose broth (TGhL) containing penicillin/streptomycin (200 mg/litre) and incubated at 15°C. Wells showing chytrid growth without bacterial contamination can be used for subculturing (Martel *et al.*, 2013). Chytrid growth can be visualised by examining the wells under an inverted microscope (10–40 × magnification).

Given the difficulties to isolate Bsal from infected animals and the high uncertainty to obtain a viable culture, this method is not appropriate as first diagnostic approach a routine diagnostic method, but (in rare cases) may be useful to confirm infection and for or to obtaining isolates for research (for example for epidemiological tracing).

4.4. Nucleic acid amplification

4.4.1. Real-time PCR

The following information is derived from Blooi *et al.* (2013), Thomas *et al.* (2018) and Sabino Pinto *et al.* (2018). DNA from skin swabs can be extracted using commercial DNA extraction kits, in 100 µl Prepman Ultra Reagent (Applied Biosystems, Foster City, CA) or by using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). ~~The latter follows the animal tissues protocol (Qiagen DNeasy Blood and Tissue kit) with pre-treatment for Gram-positive bacteria and expanded initial incubation for 1 hour.~~ DNA from skin tissue can be extracted using proteinase K digestion or DNA Easy Tissue Kit. Extracted DNA is diluted tenfold to minimise possible PCR inhibition. Controls should be run with each assay: at least a negative extraction control and a positive control; preferably, an internal PCR control is included. Positive control consists of DNA extracts of a tenfold dilution series of Bsal zoospores from 1 to 100.000 to allow quantification.

A TaqMan PCR has been partially validated to level 2 without however, stating its intended purpose (Thomas *et al.*, 2018). SYBR green real-time PCR, may be used as well but needs further validation to determine specificity and sensitivity (Martel *et al.*, 2013). The TaqMan PCR can either be used as simplex PCR or in combination with primers to detect *B. dendrobatidis* in a duplex PCR (Blooi *et al.*, 2013) and uses the forward primer STerF (5'-TGC-TCC-ATC-TCC-CCC-TCT-TCA-3'), reverse primer STerR (5'-TGA-ACG-CAC-ATT-GCA-CTC-TAC-3') and Cy5 labelled probe STerC (5'-ACA-AGA-AAA-TAC-TAT-TGA-TTC-TCA-AAC-AGG-CA-3') to detect the presence of the 5.8S rRNA gene of Bsal. Intra- and interassay efficiency were 94 and 99%, respectively (Blooi *et al.*, 2013). This

TaqMan duplex PCR does not decrease detectability of both Bd and Bsal, except in case of mixed infections (Thomas *et al.*, 2018). The use of simplex Bsal-specific PCR is therefore recommended in case Bd has been detected in the sample. The sensitivity of this real-time qPCR is between 96 and 100% and diagnostic specificity 100% (95% CI: 73–100%; Thomas *et al.*, 2018) when used in clinically affected animals. Although DNA quantities as low as 0.1 genomic equivalent can be detected (Bloo *et al.*, 2013), Thomas *et al.* (2018) recommend a threshold of 1 genomic equivalent per reaction to reduce the likelihood of false positive results. Borderline results (≤ 1 GE per reaction) should be classified as suspect and need confirmation by sequencing (or isolation).

Samples are preferably run in duplicate. A sample is considered positive based on the combination of (1) the shape of the amplification curves (2) positive results in both duplications, (3) returning GE values above the detection threshold (1 GE per reaction) (4) low variability between duplicates (< 0.3 Ct value).

4.4.2. Conventional PCR (PCR)

~~The use of real-time PCR is recommended.~~ No conventional PCR protocol has been validated.

4.4.3. Other nucleic acid amplification methods

None validated.

4.5. Amplicon sequencing

~~For confirmation of suspect samples, amplified products can be sequenced with the primers as described in 4.4.1.~~

No conventional PCR protocol has been validated.

4.6. ~~In-situ hybridisation (and histoimmunochimistry)~~

No In-situ hybridisation: no validated protocols are available.

4.7. Immunohistochemistry

Immunohistochemistry is currently not Bsal specific, due to the lack of Bsal specific antibodies (Dillon *et al.*, 2017; Thomas *et al.*, 2018). ~~Sensitivity of immunohistochemistry in diseased or dead animals can be estimated to be high if clinically affected skin regions have been selected.~~

4.8. Bioassay

Not available.

4.9. Antibody-based or antigen detection methods

A lateral flow assay (LFA) using an IgM monoclonal antibody (MAb) was developed to detect infection in amphibian skin samples. This MAb does not discriminate between *B. salamandrivorans*, *B. dendrobatidis* and *Homolaphlyctis polyrhiza* (Dillon *et al.*, 2017–2016). The sensitivity of this test is likely to be lower than that of the real-time qPCR (Dillon *et al.*, 2017): in experimentally Bd inoculated frogs, 1/5 animals tested positive in LFA compared to 4/5 using real-time qPCR. This would make this technique most useful in animals with high infection loads. Such techniques may be useful for point-of-care testing if specificity is increased and provided thorough validation.

4.10. Other serological methods

Not applicable

Annex 12 (contd)**5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations**

The use of real-time PCR on skin swabs is recommended for surveillance.

6. Corroborative diagnostic criteria

This Section only addresses the diagnostic test results for detection of infection in the ~~presence-absence~~ (Section 6.1.) or in the ~~presence-absence~~ of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent.

6.1. Apparently healthy animals or animals of unknown health status²

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

Such surveys typically consist of non-invasive sampling using skin swabs that are examined for the presence of Bsal using real-time PCR. When applied to animals in the wild, confirmation by using a complementary technique, other than sequencing the PCR product, is often not feasible.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection **with Bsal** shall be suspected if ~~a positive result has been obtained on at least one animal from at least one of the following diagnostic tests~~ criteria is met:

- i) Positive result by real-time PCR.
- ii) Histopathological changes ~~(including immunohistochemistry)~~ consistent with the presence of the pathogen or the disease.
- iii) The presence of motile spores, compatible with chytrid zoospores, in wet mount of urodele skin.

~~iv) Positive result from lateral flow assay (LFA).~~

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection **with Bsal** is confirmed if ~~positive results have been obtained on at least, in addition to the criteria in Section 6.1.1., one animal from two tests used in~~ of the following combination criteria is met:

- i) Positive result by real-time PCR, ~~on skin swab or skin tissue, and by histopathology or immunohistochemistry on skin tissue.~~
- ii) ~~Positive result by real-time PCR on skin swab or skin tissue, and~~ Pathogenic agent isolation from the skin in culture and confirmation-identification by real-time PCR.

² For example transboundary *commodities*.

6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with Bsal shall be suspected if at least one of the following criteria is met:

- i) Clinical signs (haemorrhages, ulcerations, presence of sloughed skin, see Section 2.3.2.), notably the presence of skin ulcers and/or disecdysis.
- ii) Positive result by real-time PCR, on at least one swab or skin tissue.
- iii) Histopathological changes consistent with the presence of the pathogenic agent or the disease.
- iv) Visual observation (by microscopy) of motile spores, compatible with amphibian chytrid zoospores, in a wet mount of the skin of at least one diseased urodele.
- v) Positive result of antigen detection technique such as by LFA.
- vi) Positive result from immunohistochemistry.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with Bsal is confirmed if, in addition to the criteria in Section 6.2.1., positive results have been obtained on at least one animal from two tests used in one of the following combination diagnostic tests criteria is met:

- i) Positive result by real-time PCR, on skin swab or skin tissue and by histopathology.
- ii) Positive result by real-time PCR on skin swab or skin tissue, and Pathogenic agent isolation from the skin in culture and identification by real-time PCR and confirmation by real-time PCR.

~~Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.~~

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with Bsal are provided in Table 6.3. This information can be used for the design of surveys for infection with Bsal, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

Table 6.1. Diagnostic performance of tests recommended for surveillance or diagnosis

<u>Test type</u>	<u>Test purpose</u>	<u>Source populations</u>	<u>Tissue or sample types</u>	<u>Species</u>	<u>DSe (n)</u>	<u>DSp (n)</u>	<u>Reference test</u>	<u>Citation</u>
<u>Real-time PCR</u>	<u>Diagnosis</u>	<u>Experimentally infected salamanders (clinical and subclinical infection)</u>	<u>Skin swabs</u>	<u><i>Salamandra salamandra</i></u>	<u>96–100 (26)</u>	<u>100 (12)</u>	<u>Droplet digital PCR</u>	<u>Thomas et al. (2018)</u>

DSe = diagnostic sensitivity; DSp = diagnostic specificity; n = number of samples used in the study.

Annex 12 (contd)**7. References**

BLOOI M., MARTEL A., HAESEBROUCK F., VERCAMMEN F., BONTE D. & PASMANS F. (2015a). Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. *Scientific Reports*, **5**, 8037. <https://doi.org/10.1038/srep08037>

BLOOI M., MARTEL A., VERCAMMEN F., HAESEBROUCK F. & PASMANS F. (2015b). Treatment of urodelans based on temperature dependent infection dynamics of *Batrachochytrium salamandrivorans*. *Scientific Reports*, **5**, 8037.

BLOOI M., PASMANS F., LONGCORE J.E., SPITZEN-VAN DER SLUIJS A., VERCAMMEN F. & MARTEL A. (2013). Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. *J. Clin. Microbiol.*, **51**, 4173–4177.

BLOOI M., PASMANS F., ROUFFAER L., HAESEBROUCK F., VERCAMMEN F. & MARTEL A. (2015c–2015b). Successful treatment of *Batrachochytrium salamandrivorans* infections in salamanders requires synergy between voriconazole, polymyxin E and temperature. *Scientific Reports*, **5**, 11788.

DILLON M.J., BOWKETT A.E., BUNGARD M.J., BECKMAN K.M., O'BRIEN M.F., BATES K., FISHER M.C., STEVENS J.R. & THORNTON C.R. (2017). Tracking the amphibian pathogens *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* using a highly specific monoclonal antibody and lateral-flow technology. *Microb. Biotechnol.*, **10**, 381–394.

FARRER R.A., MARTEL A., VERBRUGGHE E., ABOUELLEIL A., DUCATELLE R., LONGCORE J.E., JAMES T.Y., PASMANS F., FISHER M.C. & CUOMA C.A. (2017). Evolutionary innovations underpin niche-specific infection strategies across emerging pathogenic chytrid fungi. *Nature Communications*, **8**: 14742. doi: 10.1038/ncomms14742.

FISHER M.C., GHOSH P., SHELTON J.M.G., BATES K., BROOKES L., WIERZBICKI C., ROSA G.M., FARRER R.A., AANENSEN D.M., ALVARADO-RYBAK M., BATAILLE A., BERGER L., BÖLL S., BOSCH J., CLARE F.C., COURTOIS E., CROTTINI A., CUNNINGHAM A.A., DOHERTY-BONE T.M., GEBRESENBET F., GOWER D.J., HÖGLUND J., JAMES T.Y., JENKINSON T.S., KOSCH T.A., LAMBERTINI C., LAURILA A., LIN C.F., LOYAU A., MARTEL A., MEURLING S., MIAUD C., MINTING P., NDRIANTSOA S., O'HANLON S., PASMANS F., RAKOTONANAHARY T., RABEMANANJARA F.C.E., RIBEIRO L.P., SCHMELDER D.S., SCHMIDT B.R., SKERRATT L., SMITH F., SOTO-AZAT C., TESSA G., TOLEDO L.F., VALENZUELA-SÁNCHEZ A., VERSTER R., VÖRÖS J., WADMAN B., WEBB R.J., WELDON C., WOMBWELL E., ZAMUDIO K.R., LONGCORE J.E. & GARNER T.W.J. (2018). Development and worldwide use of non-lethal, and minimal population-level impact, protocols for the isolation of amphibian chytrid fungi. *Scientific Reports*, **8** (1), 7772. doi: 10.1038/s41598-018-24472-2.

FITZPATRICK L., PASMANS F., MARTEL A. & CUNNINGHAM A.A. (2018). Epidemiological tracing of *Batrachochytrium salamandrivorans* identifies widespread infection and associated mortalities in private amphibian collections. *Scientific Reports*, **8**, 13845.

LAKING A.E., NGO H.N., PASMANS F., MARTEL A. & NGUYEN T.T. (2017). *Batrachochytrium salamandrivorans* is the predominant chytrid fungus in Vietnamese salamanders. *Scientific Reports*, **7**, 44443. doi: 10.1038/srep44443

MARTEL A., BLOOI M., ADRIAENSEN C., VAN ROOIJ P., BEUKEMA W., FISHER M.C., FARRER R.A., SCHMIDT B.R., TOBLER U., GOKA K., LIPS K.R., MULETZ C., ZAMUDIO K., BOSCH J., LÖTTERS S., WOMBWELL E., GARNER T.W.J., SPITZEN-VAN DER SLUIJS A., SALVIDIO S., DUCATELLE R., NISHIKAWA K., NGUYEN T.T., VAN BOCXLAER I., BOSSUYT F. & PASMANS F. (2014). Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. *Science*, **346**, 630–631.

MARTEL A., SPITZEN-VAN DER SLUIJS A., BLOOI M., BERT W., DUCATELLE R., FISHER M.C., WOELTJES A., BOSMAN W., CHIERS K., BOSSUYT F. & PASMANS F. (2013). *Batrachochytrium salamandrivorans* sp nov causes lethal chytridiomycosis in amphibians. *Proc. Natl Acad. Sci. USA*, **110**, 15325–15329.

NGUYEN T.T., NGUYEN T.V., ZIEGLER T., PASMANS F. & MARTEL A. (2017). Trade in wild anurans vectors the urodelan pathogen *Batrachochytrium salamandrivorans* into Europe. *Amphibia Reptilia*, **38**, 554–556. doi: 10.1163/15685381-00003125

Annex 12 (contd)

SABINO-PINTO J., BLETZ M., HENDRIX R., PERL R.G.B., MARTEL A., PASMANS F., LÖTTERS S., MUTSCHMANN F., SCHMELLER D.S., SCHMIDT B.R., VEITH M., WAGNER N., VENCES M. & STEINFARTZ S. (2015). First detection of the emerging fungal pathogen *Batrachochytrium salamandrivorans* in Germany. *Amphibia Reptilia*, **36**, 411–416.

SABINO-PINTO J., TOBIAS KRAUSE E., BLETZ M.C., MARTEL A., PASMANS F., STEINFARTZ S. & VENCES M. (2018 2019a). Detectability vs. time and costs in pooled DNA extraction of cutaneous swabs: a study on the amphibian chytrid fungi. *Amphibia Reptilia*, **40**, 4–11 29-39.

SABINO-PINTO J., MARTEL A., PASMANS F., STEINFARTZ S. & VENCES M. (2019b). Pooling skin swabs does not inhibit qPCR detection of amphibian chytrid infection. *Plos One* **14, e0214405**

SPITZEN-VAN DER SLUIJS A., MARTEL A., ASSELBERGHS J., BALES E.K., BEUKEMA W., BLETZ M.C., DALBECK L., GOVERSE E., KERRES A., KINET T., KIRST K., LAUDELOUT A., MARIN D.A., FONTE L.F., NÖLLERT A., OHLHOFF D., SABINO-PINTO J., SCHMIDT B.R., SPEYBROECK J., SPIKMANS F., STEINFARTZ S., VEITH M., VENCES M., WAGNER N., PASMANS F. & LÖTTERS S. (2016). Expanding distribution of lethal amphibian fungus *Batrachochytrium salamandrivorans* in Europe. *Emerg. Infect. Dis.*, **22**, 1286–1288.

SPITZEN-VAN DER SLUIJS A., STEGEN G., BOGAERTS S., CANESSA S., STEINFARTZ S., JANSSEN N., BOSMAN W., PASMANS F. & MARTEL A. (2018). Post-epizootic salamander persistence in a disease-free refugium suggests poor dispersal ability of *Batrachochytrium salamandrivorans*. *Scientific Reports*, **8**, 3800.

STEGEN G., PASMANS F., SCHMIDT B.R., ROUFFAER L.O., VAN PRAET S., SCHAUB M., CANESSA S., LAUDELOUT A., KINET T., ADRIAENSEN C., HAESBROUCK F., BERT W., BOSSUYT F. & MARTEL A. (2017). Drivers of *Batrachochytrium salamandrivorans* mediated salamander extirpation. *Nature*, **544**, 353–356. doi: 10.1038/nature22059

THOMAS V., BLOOI M., VAN ROOIJ P., VAN PRAET S., VERBRUGGHE E., GRASSELLI E., LUKAC M., SMITH S., PASMANS F. & MARTEL A. (2018). Recommendations on diagnostic tools for *Batrachochytrium salamandrivorans*. *Transbound. Emerg. Dis.*, **65**, e478-e488.

VAN ROOIJ P., MARTEL A., HAESBROUCK F. & PASMANS F. (2015). Amphibian chytridiomycosis: a review with focus on fungus-host interactions. *Vet. Res.*, **46**, 137.

VAN ROOIJ P., PASMANS F., COEN Y. & MARTEL A. (2017). Efficacy of chemical disinfectants for the containment of the salamander chytrid fungus *Batrachochytrium salamandrivorans*. *PLoS One*, **12**, e0186269. doi: 10.1371/journal.pone.0186269

WHITE L.W., FORZÁN M.J., PESSIER A.P., ALLENDER M.C., BALLARD J.R., CATENAZZI A., FENTON H., MARTEL A., PASMANS F., MILLER D.L., OSSIBOFF R.J., RICHGELS K.L.D. & KERBY J.L. (2016). Amphibian: a case definition and diagnostic criteria for *Batrachochytrium salamandrivorans* chytridiomycosis. *Herpetological Review*, **47**, 207.

YAP T.A., KOO M.S., AMBROSE R.F., WAKE D.B. & VREDENBURG V.T. (2015). BIODIVERSITY: Averting a North American biodiversity crisis. *Science*, **349**, 481–482.

ZHIYONG Y., MARTEL A., WU J., VAN PRAET S., CANESSA S. & PASMANS F. (2018). Widespread occurrence of an emerging fungal pathogen in heavily traded Chinese urodelan species. *Conservation Letters*, **11**: e12436.

*
* *

NB: There are currently no OIE Reference Laboratories for infection with *Batrachochytrium salamandrivorans*
NB: First adopted in 2020.

CHAPTER 2.3.4.

INFECTION WITH INFECTIOUS HAEMATOPOIETIC NECROSIS VIRUS

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter.

One comment inserted within the text below.

1. Scope

Infection with infectious haematopoietic necrosis virus means infection with the pathogenic agent Salmonid novirhabdovirus (commonly known as infectious haematopoietic necrosis virus [IHNV]) of the Genus *Novirhabdovirus* and Family *Rhabdoviridae*.

2. Disease information

2.1. Agent factors

2.1.1. Aetiological agent

IHNV consists of a bullet-shaped particle of approximately 150–190 nm in length and 65–75 nm in diameter that encapsulates a non-segmented, negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides. The viral genome codes for six proteins in the following order: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a glycoprotein (G), a non-virion protein (NV), and a polymerase (L). Due to the primary position of the nucleoprotein gene on the IHNV genome, nucleoprotein transcripts and protein are the first and most abundant during viral infection and is typically the preferred target of diagnostic tests. The glycoprotein forms spike-like projections on the surface of the mature virion and is the primary antigenic component of the virus such that anti-glycoprotein serum is sufficient to neutralise infections.

The type strain of IHNV is the Western Regional Aquaculture Center (WRAC) strain available from the American Type Culture Collection (ATCC VR-1392). The GenBank accession number of the genomic sequence of the WRAC strain is L40883 (Morzunov *et al.*, 1995; Winton & Einer-Jensen, 2002).

Phylogenetic analyses based on G-gene nucleotide sequences have classified IHNV isolates into five major genogroups denoted U, M, L, E, and J that correspond to geographical location rather than host species (Cieslak *et al.*, 2017; Enzmann *et al.*, 2005; 2010; Johansson *et al.*, 2009; Kim *et al.*, 1999; Kolodziejek *et al.*, 2008; Kurath *et al.*, 2003; Nishizawa *et al.*, 2006). Nevertheless, IHNV displays a strong phylogeographic signature reflecting the host species from which the virus is most commonly isolated in various geographical areas (e.g. sockeye salmon [*Oncorhynchus nerka*] in the Northeast Pacific – U genogroup; Chinook salmon [*Oncorhynchus tshawytscha*] in California, USA – L genogroup; and rainbow trout [*Oncorhynchus mykiss*] in Europe, Asia, and Africa (Mulei *et al.*, 2019) and Idaho, USA – E, J and M genogroups, respectively). Additionally, experimental infections demonstrating that U and M genogroup viruses had higher virulence in sockeye salmon and rainbow trout, respectively (Garver *et al.*, 2006), supports the observation that IHNV strains isolated from its historical phylogeographic host tends to be more virulent for the same species in comparison to other species.

2.1.2. Survival and stability in processed or stored samples

IHNV stability in host tissues during storage and processing is largely influenced by temperature. The virus is more stable at lower temperature and remained infectious for at least 3 days at 4°C in naturally infected or IHNV-seeded tissue (Burke & Mulcahy, 1983; Gosting & Gould, 1981; Hostnik *et al.*, 2002; Pietsch *et al.*, 1977). For long-term survival of infectious virus, tissues should be stored at temperatures below –20°C (Burke & Mulcahy, 1983; McClure *et al.*, 2008). The preferred method for retaining infectious virus is to maintain the IHNV sample on ice with rapid processing and inoculation of cell cultures as soon as possible

due to the progressive reduction in titre with increasing temperature (Barlic-Maganja *et al.*, 2002; Gosting & Gould, 1981).

2.1.3. Survival and stability outside the host

IHNV can survive outside the host tissue in fresh water and sea water, but is impacted-affected by temperature, ultraviolet (UV) exposure, microbial community and suspended sediments. At 4°C–15°C, 10⁶ pfu/ml of IHNV remained detectable via cell culture after 1 week in either fresh or salt water (Kell *et al.*, 2014). For all genotypes, inactivation rates are reduced at lower water temperatures and virions remain infectious for longer in freshwater compared with seawater (Kell *et al.*, 2014). However, when exposed to sunlight (UV-A and UV-B), IHNV at the water surface is rapidly inactivated with six orders of magnitude of virus rendered non-infectious within 3 hours (Garver *et al.*, 2013). In addition, infectious virus is inactivated by the microbial community within the water source and with increased amounts of suspended sediments (Garver *et al.*, 2013; Kamei *et al.*, 1987).

For inactivation methods, see Section 2.4.6.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with IHNV according to Chapter 1.5 of *Aquatic Animal Health Code (Aquatic Code)* are:

Family	Scientific name	Common name
Esocidae	<i>Esox lucius</i>	Northern pike
Salmonidae	<i>Salmo marmoratus</i>	Marble trout
	<i>Salmo salar</i>	Atlantic salmon
	<i>Salmo trutta</i>	Brown trout
	<i>Salvelinus alpinus</i>	Arctic char
	<i>Salvelinus fontinalis</i>	Brook trout
	<i>Salvelinus namaycush</i>	Lake trout
	<i>Oncorhynchus clarki</i>	Cutthroat trout
	<i>Oncorhynchus tshawytscha</i>	Chinook salmon
	<i>Oncorhynchus keta</i>	Chum salmon
	<i>Oncorhynchus kisutch</i>	Coho salmon
	<i>Oncorhynchus masou</i>	Maseu salmon
	<i>Oncorhynchus mykiss</i>	Rainbow trout
	<i>Oncorhynchus nerka</i>	Sockeye salmon

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with IHNV according to Chapter 1.5 of the *Aquatic Code* are: White sturgeon (*Acipenser transmontanus*), European eel (*Anguilla anguilla*), Tube-snout (*Aulorhynchus flavidus*), Pacific herring (*Clupea pallasii*), Shiner perch (*Cymatogaster aggregate*) and Turbot (*Scophthalmus maxima*).

Family	Scientific name	Common name
Acipenseridae	<i>Acipenser transmontanus</i>	White sturgeon
Anguillidae	<i>Anguilla anguilla</i>	European eel
Aulorhynchidae	<i>Aulorhynchus flavidus</i>	Tube-snout
Clupeidae	<i>Clupea pallasii</i>	Pacific herring
Embiotocidae	<i>Cymatogaster aggregate</i>	Shiner perch
Scophthalmidae	<i>Scophthalmus maxima</i>	Turbot

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: Common carp (*Cyprinus carpio*) and American yellow perch (*Perca flavescens*).

Family	Scientific name	Common name
Cyprinidae	<i>Cyprinus carpio</i>	Common carp
Percidae	<i>Perca flavescens</i>	American yellow perch

2.2.3. Non-susceptible species

None known.

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

IHNV predominantly infects salmon and trout salmonid species with fry being the most highly susceptible stage (LaPatra, 1998). Resistance to infection typically increases with fish age until the spawning stage. Returning adult spawning salmon, can be highly infected and shed large amounts of virus in ovarian fluid and milt despite a lack the absence of clinical disease (Dixon *et al.*, 2016).

2.2.5. Distribution of the pathogen in the host

IHNV targets haematopoietic tissue and is most commonly isolated from kidney and spleen tissues. The virus has also been isolated from gill, oesophagus, intestine, stomach, pyloric caeca, liver, brain, heart, thymus, adipose tissue, muscle, skin, fin and mucous (Drolet *et al.*, 1994; Harmache *et al.*, 2006; LaPatra *et al.*, 1989; Yamamoto *et al.*, 1990). In spawning fish IHNV has also been isolated in ovarian fluid and milt (Mulcahy *et al.*, 1982).

2.2.6. Aquatic animal reservoirs of infection

Field surveillance programmes and experimental infection trials have documented subclinical IHNV infections in various salmon and trout species (Knusel *et al.*, 2007; Mulcahy *et al.*, 1984; Pascoli *et al.*, 2015; St-Hilaire *et al.*, 2001; Traxler *et al.*, 1997). Survivors of laboratory exposures have demonstrated IHNV persistence for months to over one-year post-exposure (Drolet *et al.*, 1995; Foott *et al.*, 2006; Kim *et al.*, 1999; Muller *et al.*, 2015). With the exception of high viral load occurring in subclinically infected spawning adult salmon, the IHNV levels associated with subclinical infections tend to be lower than in fish undergoing clinical disease.

2.2.7. Vectors

A single study has demonstrated that adult salmon lice, *Lepeophtheirus salmonis* are capable of acquiring and transmitting IHNV to naïve Atlantic salmon through parasitism (Jakob *et al.*, 2011). Regardless of whether salmon lice acquired IHNV through water bath exposure or after parasitising IHNV-infected fish, the duration of virus association with salmon lice diminished rapidly with infectious virus levels falling below cell culture detection limits within hours. IHNV has also been isolated from freshwater invertebrates (e.g. leeches, copepods, and mayflies), however, their capacity to transmit virus is unknown (Dixon *et al.*, 2016; Garver & Wade, 2017).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Depending on the species of fish, rearing conditions, temperature, and virus strain, outbreaks of infection with IHNV may range from acute to chronic. An outbreak of infection with IHNV in farmed Atlantic salmon in British Columbia resulted in cumulative losses on affected farms between of 20 and 94% (Saksida, 2006). In chronic cases, losses are protracted and fish in various stages of disease can be observed in the pond. The prevalence of infection in chronic cases remains unknown. The limited available data indicated that prevalence of infection with IHNV can be high (59%) in endemically infected rainbow trout farms in Europe (reviewed by Dixon *et al.*, 2016).

IHNV is endemic among populations of free-ranging salmonids throughout much of its historical range along the west coast of North America. Sockeye salmon have incurred losses of up to 99% 36.9% at the fry stage (Kurath *et al.*, 2003; Meyers *et al.*, 2003). As the fish With ages, the prevalence of infection decreases with in marine phase sockeye salmon smolts, and the prevalence of infection in adults is generally low (<15%) to undetectable. However, the prevalence of infection can again reach high levels in mature adult spawning sockeye salmon, with long-term studies revealing greater than 50% prevalence in wild populations (Meyers *et al.*, 2003).

UNOFFICIAL VERSION

Annex 13 (contd)

2.3.2. Clinical signs, including behavioural changes

Fish with acute infection with IHNV can exhibit lethargy interspersed with bouts of frenzied, abnormal activity. During outbreaks, fish can display spiral swimming, flashing, and have trailing faecal casts. Fish may also show darkening of the skin, exophthalmia, distended abdomen and external haemorrhaging. In instances where fish survive an outbreak, spinal deformities may become evident (Bootland & Leong, 1999).

2.3.3 Gross pathology

Gross observations are non-pathognomonic and **can involve may include** ascites, pale gills, liver, kidney and spleen, petechial haemorrhaging, yellow mucous in the intestine and a lack of food in the stomach (Bootland & Leong, 1999; Traxler, 1986).

2.3.4. Modes of transmission and life cycle

The transmission of IHNV between fish is primarily horizontal through direct contact with virus contaminated water or via cohabitation with IHNV infected fish (Bootland & Leong, 1999). **However, cases of vertical or egg-associated transmission have been recorded (Mulcahy & Pascho, 1985). There is insufficient evidence to demonstrate true vertical transmission. Outbreaks of IHNV as a result of egg movements likely occurred as a result of inadequate disinfection of moderately infected or untested broodstock (Dixon *et al.*, 2016). While egg-associated transmission is significantly reduced by the now common practice of surface disinfection of eggs with an iodophor solution, it is the only mechanism accounting for the appearance of infection with IHNV in new geographical locations among fry originating from eggs that were incubated and hatched in virus-free water (Dixon *et al.*, 2016; Winton, 1991).**

EU comment

On the issue of vertical transmission of IHNV, the evidence suggests egg-associated transmission related to the infection of the egg surfaces when the ova or milt are infected, and the process of disinfection at collection and/or after incubation are not effective. For this reason we suggest rewording the above sentence as follows:

“There is insufficient evidence to demonstrate true vertical transmission. Outbreaks of IHNV as a result of egg movements likely occurred as a result of inadequate disinfection of eggs originating from moderately infected or untested broodstock”.

2.3.5. Environmental and management factors

The most important environmental factor affecting the disease progression is water temperature. Experimental trials have demonstrated that IHNV can produce mortality **in water temperatures** from 3°C to 18°C; however clinical disease typically occurs below 15°C under natural conditions (LaPatra, 1998).

2.3.6. Geographical distribution

Cases of infection with IHNV have been reported from Europe, Asia-Pacific, **Africa** and the Americas. For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Plasmid DNA vaccines containing the gene for the IHNV glycoprotein have proven highly efficacious against infection with IHNV resulting in the licensing of one for commercial use in Atlantic salmon net-pen aquaculture on the west coast of North America (Alonso & Leong, 2013; Salonijs *et al.*, 2007). Administered via intramuscular injection, an IHNV DNA vaccine was rapidly disseminated systemically followed by plasmid persistence in muscle at the injection site (**Garver *et al.*, 2005**); consequently, caution

should be employed when testing fish vaccinated with the IHNV DNA vaccine as diagnostic methods targeting viral G-gene nucleotide sequence or protein have the potential to cross react with the vaccine.

2.4.2. Chemotherapy including blocking agents

Chemotherapeutics, including natural compounds, have been identified to have anti-IHNV properties; however, these have not found commercial use in aquaculture against IHNV (Winton, 1991). Direct application of anti-IHNV compounds to cell cultures has caused growth inhibition and toxicity that could affect the sensitivity of detecting IHNV in affected cultures (Balmer *et al.*, 2017; Hasobe & Saneyoshi, 1985).

2.4.3. Immunostimulation

Immunostimulants are not used commercially in aquaculture for IHNV (Ooi *et al.*, 2008).

2.4.4. Breeding resistant strains

Experimental trials of triploid or inter-species hybrids have been conducted (Barroso *et al.*, 2008; Winton, 1991) with resistance typically determined early in the infection process and associated with lower early viral replication (Purcell *et al.*, 2010). However, no resistant strains are commercially available.

2.4.5. Inactivation methods

IHNV is readily inactivated by common disinfectants with active ingredients such as sodium hypochlorite, iodophor, benzalkonium chloride, saponated cresol, formaldehyde and potassium permanganate solution (Yoshimizu *et al.*, 2005). As these substances have virucidal properties any carry-over on sampling equipment or contact with samples may result in reduced viral titres.

2.4.6. Disinfection of eggs and larvae

Iodophor disinfection of eggs is a common practice to effectively mitigate egg-associated transmission of IHNV (Bovo *et al.*, 2005). Chapter 4.4. of the *Aquatic Code* provides recommendations for surface disinfection of salmonid eggs. Iodine has been shown to inhibit PCRs (Auinger *et al.*, 2008) and could affect PCR testing results of disinfected eggs.

2.4.7. General husbandry

In addition to disinfection of eggs (according to Chapter 4.4 of the *Aquatic Code*), use of a virus-free water supply and decreasing rearing densities have significant positive effects in the management of IHNV. Transmission of IHNV increases with host density (Ogut & Reno, 2004).

3. Specimen selection, sample collection, transportation and handling

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples which are most likely to be infected.

3.1. Selection of populations and individual specimens

Clinical inspections are best should be carried out during a period when ever the water temperature is below 14°C, or whenever the water temperature is likely to reach its lowest annual point. All production units (ponds, tanks, net-cages, etc.) must should be inspected for the presence of dead, weak or abnormally behaving fish of any susceptible species, and if they are present, such fish should be selected. Particular attention should be paid to the water outlet area, where weak fish tend to accumulate due to the water current.

For the purposes of disease surveillance, fish to be sampled are selected as follows: If additional fish are required for the sample, healthy individuals should be selected as follows:

- i) Rainbow trout and the other susceptible species listed in Section 2.2.1 should be sampled proportionally, or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status). In farms with salmonids, if rainbow trout are present, only fish of that species should be selected for sampling. If rainbow trout are not present, the sample has to be obtained from fish of all other IHNV-susceptible species.

- ii) Susceptible species should be sampled following risk-based criteria for targeted selection of populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown risk status).
- ii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.
- iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

For disease outbreak investigations, moribund fish or fish exhibiting clinical signs of infection with IHNV should be collected. Ideally fish should be collected while alive, however recently dead fish can also be selected for diagnostic testing. It should be noted however, that there will be a significant risk of contamination with environmental bacteria if the animals have been dead for some time. There may be no clinical signs or gross pathognomonic lesions in cases of sudden mortality.

3.2. Selection of organs or tissues

The optimal tissues material to be examined is are spleen, anterior kidney, and either heart or brain. In the case of spawning fish, ovarian fluid and milt may be taken-examined.

In the case of small fry, whole fish less than 4 cm long can be homogenised (using, for example, sterile scissors or a scapel) after removal of the body behind the anal pore-gut opening. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney should be collected. For larger size fish, kidney, spleen, heart, encephalon, and ovarian fluid from brood fish at the time of spawning, should be the tissues to be sampled. When possible, Samples should be taken in duplicate to permit retesting if needed.

3.3. Samples or tissues not suitable for pathogen detection

IHNV is very sensitive to enzymic degradation, therefore sampling tissues with high enzymatic activities or large numbers of contaminating bacteria, such as the intestine or skin, should be avoided when possible. Given the haematopoietic nature of IHNV, muscle tissue should be avoided as a target tissue. The yolk sac of fry has also shown toxicity to cell lines and should be removed before inoculating cells for virus isolation. Preservatives and fixatives, such as RNAlater and formaldehyde can be toxic to tissue culture cells such as epithelioma papulosum cyprini (EPC) and fathead minnow (FHM), and can impact molecular detection methods (Auinger *et al.*, 2008; Pham *et al.*, 2018).

3.4. Non-lethal sampling

Ovarian fluid and milt are suitable samples for detection of IHNV in spawning adult salmon and trout (Dixon *et al.*, 2016; Meyers *et al.*, 2003). There is evidence that IHNV may be isolated from gill, fin and mucous samples but detection may be impacted by the state of infection, time since exposure and sample size (Burbank *et al.*, 2017; LaPatra *et al.*, 1989).

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

3.5.1. Samples for pathogen isolation

The success of pathogen isolation and results of bioassay depend strongly on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternate storage methods should only be used after consultation with the receiving laboratory.

Before shipment or transfer to the laboratory, pieces of the organs to be examined should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing transport medium, i.e. cell culture medium with 10% fetal calf serum (FCS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficacy may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g) and taken in duplicate if retesting may be required.

Tubes containing fish tissues in transport medium for cell cultivation should be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples should be avoided. The temperature of a sample during transit must never exceed 10°C, and ice must still be present in the transport box at receipt or **at least** one **or more** freeze blocks **s** must still be partly or completely frozen.

UNOFFICIAL VERSION

Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory. All packaging and labelling must be performed in accordance with present national and international transport regulations, as appropriate.

The virological examination on cell culture should be started as soon as possible, and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium, and that the temperature requirements during transportation can be fulfilled.

3.5.2. Preservation of samples for molecular detection

Samples can be taken from the fish in accordance with the procedure described in Section 3.5.1., using a sterile instrument, and transferred to a sterile plastic tube containing transport medium.

Alternatively, samples may be placed in at least five volumes of RNA stabilisation reagents, according to the recommendation from the manufacturers. Samples in RNA stabilising reagents can be shipped on ice or at room temperature if transport time does not exceed 24 hours.

Whole fish may also be sent to the laboratory (see Section 3.5.1).

3.5.3. Fixed samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Tissue samples for histopathology should be immediately fixed at a fixative to tissue ratio of 10:1. A suitable fixative is 10% buffered formalin. To avoid excessive cross-linking, tissue should be transferred to ethanol after 24hrs if methods other than histopathology are used e.g. *in-situ* hybridisation.

3.5.4. Fixed samples for electron microscopy

Not relevant. Samples for electron microscopy are not routinely required and are collected only when it is considered beneficial to facilitate further diagnostic investigation. A 2 mm cubed section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.

3.5.5. Samples for other tests

Not relevant.

3.6. Pooling of samples

No data are currently available concerning the effect of pooling samples on the detection of IHNV. However, small life stages such as fry can be pooled to provide the minimum amount of material needed for testing. Pooling of samples from more than one individual animal for a given purpose should only be recommended where supporting data on diagnostic sensitivity and diagnostic specificity are available. However, smaller life stages (e.g. fry) can be pooled to provide a minimum amount of material for testing.

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

- +++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;
- ++ = Suitable method(s) but may need further validation;
- + = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;

Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of **apparently** healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juvenile s ²	Adults	LV	Early life stages ²	Juvenile s ²	Adults	LV
Wet mounts												
Histopathology ³						++	++	1				
Cytopathology ³												
Cell or artificial media culture	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Real-time PCR	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Conventional PCR						++	++	++ ²	++	++	++	<u>1</u>
Amplicon sequencing ⁴									+++	+++	+++	<u>3</u>
<i>In-situ</i> hybridisation												
Bioassay												
LAMP												
IFAT									++	++	++	2
Ag-ELISA									++	++	++	2
Neutralisation test (antibody or antiserum) ⁵									++	++	++	2

LV = level of validation, refers to the stage of validation in the OIE Pathway (Chapter 1.1.2); PCR = polymerase chain reaction; LAMP = loop-mediated isothermal amplification. IFAT = indirect fluorescent antibody test; Ag-ELISA = antigen enzyme-linked immunosorbent assay. ¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Early and juvenile life stages have been defined in Section 2.2.4.

³Cytopathology and histopathology can be validated if the results from different operators has been statistically compared.

⁴Sequencing of the PCR product.

⁵Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Not relevant

4.2. Histopathology and cytopathology

Histopathological findings reveal degenerative necrosis in haematopoietic tissues, kidney, spleen, liver, pancreas, and digestive tract. Necrosis of eosinophilic granular cells in the intestinal wall is pathognomonic of IHNV infection (Bootland & Leong, 1999).

The blood of affected fry shows reduced haematocrit, leukopenia, degeneration of leucocytes and thrombocytes, and large amounts of cellular debris. As with other haemorrhagic viraemias of fish, blood chemistry is altered in severe cases (Bootland & Leong, 1999).

Electron microscopy of virus-infected cells reveals bullet-shaped virions of approximately 150–190 nm in length and 65–75 nm in width (Wolf, 1988). The virions are visible at the cell surface or within vacuoles or intracellular spaces after budding through cellular membranes. The virion possesses an outer envelope containing host lipids and the viral glycoprotein spikes that react with immunogold staining to decorate the virion surface

Smears are not appropriate for detection or identification of IHNV.

4.3. Cell or artificial media culture for isolation

4.3.1. Cell lines

The recommended cell lines for IHNV detection are EPC or FHM. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

EPC or FHM cells are grown at 20–30°C in suitable medium, e.g. Eagle's minimal essential medium (MEM; or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris-HCl (23 mM) and Na-bicarbonate (6 mM). The pH must be 7.6 ± 0.2 . Cell culture plates should be seeded 4–48 hours and not 100% confluent prior to inoculation. 15–30 minutes prior to sample inoculation, cells should be pre-treated with 7% (w/v) PEG-20,000 solution ($10\text{--}15 \mu\text{l}/\text{cm}^2$) (Batts & Winton, 1989).

4.3.2. Sample preparation and inoculation

Note: Tissue and fluid samples should be kept cool throughout sample preparation procedures.

- i) Homogenise tissue samples using mortar and pestle ~~or a tissue homogeniser, stomacher, polytron or equivalent~~. A small volume of media (MEM-4 or Hank's balanced salt solution with antibiotics) may be needed to achieve complete homogenisation.
- ii) Adjust the volume of media to a final ratio of 10:1 (media:tissue) and mix thoroughly. For fluid samples adjust the volume of media to a final ratio of 1:1.
- iii) Centrifuge the homogenate or fluid samples at 2000–4000 g for 15 minutes at 2–5°C.
- iv) Remove the supernatant and pass through a 0.45 µm membrane filter (if available).
- v) If the sample cannot be inoculated within 48 hours after collection, the supernatant may be stored at –80°C provided virological examination is carried out within 14 days.
- vi) If samples originate from an area where infectious pancreatic necrosis virus (IPNV) is present, supernatants may be treated with IPNV antiserum. Mix the supernatant with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPNV and incubate for a minimum of 1 hour at 15°C or up to 18 hours at 4°C. The titre of the antiserum must be at least 1/2000 in a 50% plaque neutralisation test.

Annex 13 (contd)

- vii) Samples are inoculated into cell cultures in at least two dilutions, i.e. the primary dilution and a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively. The ratio between inoculum size and volume of cell culture medium should be about 1:10. For each dilution and each cell line, a minimum of about 2 cm² cell area, corresponding to one well in a 24-well cell culture tray, has to be used. Use of cell culture trays is recommended, but other units of similar or with larger growth area are acceptable as well.
- viii) Inoculated cell cultures are incubated at 15°C for 7–10 days. Using a microscope with 40–150× magnification, cultures should be inspected for toxicity the day after inoculation, particularly if supernatant was not filtered in step iv. The use of a phase-contrast microscope is recommended.
- ix) ~~Monitor the~~ The cells **are monitored** regularly (2–3 times a week) for the presence of cytopathic effect (CPE).

Interpretation of results

If CPE is observed, confirmatory testing is required to identify IHNv.

If no CPE is observed in the primary culture or subcultivation, the sample is negative.

4.4. Nucleic acid amplification**4.4.1. Real-time PCR**

There are several ~~reverse-transcription~~ real-time **reverse-transcription (RT)** PCR assays available for the detection of IHNv. **Two assays are described, a two-step real-time PCR and a one-step real-time PCR.**

The **first** assay described is a stage 3 validated two-step real-time TaqMan PCR assay that amplifies a region of the nucleoprotein gene of all known IHNv genogroups with some E-genogroup isolates (D332-92, FV23, and FV91-40) having reduced amplification efficiency due to single nucleotide polymorphism within the probe sequence (Hoferer *et al.*, 2019; Purcell *et al.*, 2013).

Positive and negative controls should be run with each stage of the assay: extraction, reverse transcription and real-time PCR. Due to the sensitive nature of PCR-based assays, it is important to be able to distinguish a true positive from the positive control material. This may be achieved using an artificial positive control as employed by Purcell *et al.* (2013). It is also highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.

RNA extraction and reverse-transcription (RT)

- i) Total RNA from infected cells and/or tissues is extracted using a phase-separation method (e.g. phenol-chloroform or Trizol) or by use of a commercially available RNA isolation kit used according to the manufacturer's instructions.
- ii) Extracted RNA is reverse transcribed non-discriminately into cDNA using random primers. The cDNA synthesis reactions and cycling conditions are best performed using the manufacturer's instructions for commercially available kits which have been extensively tested with a variety of RNA templates, including GC- and AU-rich targets and RNase expressed at low levels.

Real-time PCR

The TaqMan real-time PCR assay uses forward primer IHNv N 796F (5'-AGA-GCC-AAG-GCA-CTG-TGC-G-3'), reverse primer IHNv N 875R (5'-TTC-TTT-GCG-GCT-TG-GTT-GA-3') and FAM-labelled probe, IHNv N 818T (5'-6FAM-TGA-GAC-TGA-GCG-GGA-CA-MGBNFQ-3'). Primers are used at a final concentration of 900 nM each and the final probe concentration is 250 nM. 2.5 µl cDNA product is added to each 25 µl rPCR reaction. Thermal cycling conditions are 50°C 2 minutes, 95°C 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The sample is negative if no Ct (threshold cycle) is recorded, while samples with a Ct are considered positive for IHNv.

One step real-time RT-PCR

The one step real-time RT-PCR is performed using the SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific, Schwerte, Germany) and/or the AgPath-ID One-Step RT-PCR Kit (Thermo Fisher Scientific) according to the manufacturers' instructions. For all quantitative assays, the following unique parameters were used: (a) total volume of 25 µl consisting of 20 µl mastermix and 5 µl of RNA; (b) 900 nM of each primer; (c) 200 nM of IHNV probe and 250 nM of VHSV probe, respectively; (d) hard-shell 96-well skirted plates with white shell (Bio-Rad, Munich, Germany, cat. No HSP9601); (e) Microseal B adhesive optical clear seals (Bio-Rad, cat. no MSB 1001); (f) run on a C1000TM Thermal Cycler controlled by the CFX96TM Real-Time PCR Detection System (Bio-Rad); and (g) use of the CFX Manager software (Bio-Rad) for data analysis. The threshold was set automatically (Hoferer *et al.*, 2019)

4.4.2. Conventional PCR

Several conventional PCR assays are available with limited validation data.

The PCR assay described recognises a broad range of genotypes by targeting a central region of the IHNV G gene (Emmenegger *et al.*, 2000), and produces a PCR amplicon that is used for identification of genetic strains and for epidemiological tracing of virus movements (Kurath *et al.*, 2003).

Positive and negative controls should be run with each stage of the assay: extraction, RT-PCR and second round PCR. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.

RNA extraction

Total RNA may be prepared as described in section 4.4.1.

Conventional RT-PCR (Round 1)

The first round RT-PCR combines cDNA synthesis and PCR amplification into one step by using an IHNV-specific primer set that generates the first-strand synthesis of IHNV RNA and subsequent PCR amplification through 30 cycles. The first round PCR produces a 693 bp PCR amplicon using forward primer (5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3') and reverse primer (5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3') at a final concentration of 200 nM each. The thermal cycling conditions are one cycle of 50°C for 30 minutes; one cycle of 95°C for 2 minutes; 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; one cycle of 72°C for 7 minutes and 4°C hold.

A sample is IHNV positive if a 693 bp PCR amplicon is observed and no bands were observed in the negative controls. If no band is observed for a sample and the positive controls passed proceed to the second round nested PCR.

Second round (nested PCR)

Due to the sensitivity of the test along with the need for repetitive handling of tubes, nested PCR is prone to contamination and good sterile technique must be practiced.

The first round positive and negative controls are carried over and included with the nested PCR assay. In addition, a separate negative and positive control specific to the nested assay are required.

The second round PCR produces a 483 bp PCR amplicon using forward primer (5'-TCA-CCC-TGC-CAG-ACT-CAT-TGG-3') and reverse primer (5'-ATA-GAT-GGA-GCC-TTT-GTG-CAT-3') at a final concentration of 200 nM each. The thermal cycling conditions are: 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds; one cycle of 72°C for 7 minutes and 4°C hold.

A sample is IHNV positive if a 483 bp PCR amplicon is observed and no band(s) are observed in the negative controls. A sample is negative if no bands are observed and positive controls passed.

Annex 13 (contd)**4.4.3. Other nucleic acid amplification methods**

To date, no other nucleic acid amplification method capable of universal IHNV detection has been sufficiently validated.

4.5. Amplicon sequencing

Nucleotide sequencing of the conventional PCR product (Section 4.4.2) is recommended as one of the final steps for confirmatory diagnosis. This central region of IHNV glycoprotein gene is used for identification of genetic strains and for epidemiological study (Kurath *et al.*, 2003). It is recommended to forward any sequence data obtained to the OIE Reference Laboratory, particularly in the event where isolate sequences differ from any of the target sequences of the recommended molecular assays.

4.6. *In-situ* hybridisation

Not relevant.

4.7. Immunohistochemistry

Not relevant.

4.8. Bioassay

Not relevant.

4.9. Antibody- or antigen-based detection methods

Antibody- and antigen-based detection methods may be used to confirm the presence of IHNV in cell culture. Kits and antibodies are commercially available and should be used according to manufacturer's instructions. Sensitivity, specificity and sample preparation can influence the results; a negative result should be viewed with caution. These techniques should not be used as a screening method.

4.9.1. Neutralisation test (identification in cell culture)

- i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge an aliquot at 2000 g for 15 minutes at 4°C, or filter through a 0.45 µm (or 450 nm) pore membrane to remove cell debris.
- ii) Dilute virus-containing medium from 10²–10⁴.
- iii) Mix aliquots (for example 200 µl) of each dilution with equal volumes of an IHNV antibody solution. The neutralising antibody solution must have a 50% plaque reduction titre of at least 2000. Likewise, treat a set of aliquots of each virus dilution with cell culture medium to provide a non-neutralised control.
- iv) In parallel, a neutralisation test must be performed against a homologous IHNV strain (positive neutralisation test) to confirm the reactivity of the antiserum.
- v) Incubate all the mixtures at 15°C for 1 hour.
- vi) Transfer aliquots of each of the above mixtures on to 24-hour-old monolayers overlaid with cell culture medium containing 10% FBS (inoculate two wells per dilution) and incubate at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.
- vii) Check the cell cultures for the onset of CPE and read the results for each suspect IHNV sample and compare to the occurrence of CPE of non-neutralised controls. Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding the cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.
- viii) The tested virus is identified as IHNV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the IHNV-specific antibody, whereas CPE is evident in all other cell cultures.

Other neutralisation tests of demonstrated performance may be used instead.

4.9.2. Indirect fluorescent antibody test (IFAT) (identification in cell culture)

- i) Prepare monolayers of cells in 2 cm² wells of cell culture plastic plates or on cover slips in order to reach around 80% confluency, which is usually achieved within 24 hours of incubation at 22°C—the optimal temperature of the cell line in question (e.g. 26°C for EPC and 20°C for RTG) (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FBS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of black 96-well plates for immunofluorescence is recommended.
- ii) When the cell monolayers are ready for infection (i.e. on the same day or on the day after seeding) inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.
- iii) Dilute the control virus suspension of IHNV in a similar way, in order to obtain a virus titre of about 5,000–10,000 plaque-forming units (PFU)/ml in the cell culture medium.
- iv) Incubate at 15°C for 24 hours.
- v) Remove the cell culture medium, rinse once with 0.01 M phosphate buffered saline (PBS), pH 7.2, then three times briefly with a cold mixture of acetone 30%/ethanol 70% (v/v) (stored at –20°C).
- vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm² of cell monolayer.
- vii) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at –20°C.
- viii) Prepare a solution of purified IHNV antibody or serum in 0.01 M PBS, pH 7.2, containing 0.05% Tween-80 (PBST), at the appropriate dilution (which has been established previously or is given by the reagent supplier).
- ix) Rehydrate the dried cell monolayers by four rinsing steps with the PBST solution and remove this buffer completely after the last rinsing.
- x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur (e.g. by adding a piece of wet cotton to the humid chamber). The volume of solution to be used is 0.25 ml/2 cm² well.
- xi) Rinse four times with PBST as above.
- xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate- or tetramethylrhodamine-5-(and-6-) isothiocyanate-conjugated antibody to the immunoglobulin used in the first layer and prepared according to the instructions of the supplier. These conjugated antibodies are most often rabbit or goat antibodies.
- xiii) Rinse four times with PBST.
- xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover slips using, for example, glycerol saline, pH 8.5 prior to microscopic observation.
- xv) Examine under incident UV light using a microscope with × 10 eye pieces and × 20–40 objective lens having numerical aperture >0.65 and >1.3, respectively. Positive and negative controls must be found to give the expected results prior to any other observation.

Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of demonstrated performance may be used instead.

4.9.3. Enzyme-linked immunosorbent assay (ELISA)

- i) Coat the wells of microplates designed for ELISAs with appropriate dilutions of purified immunoglobulins (Ig) or serum specific for IHNV, in 0.01 M PBS, pH 7.2 (200 µl/well).
- ii) Incubate overnight at 4°C.
- iii) Rinse four times with 0.01 M PBS containing 0.05% Tween-20 (PBST).
- iv) Block with skim milk (5% in PBST) or other blocking solution for 1 hour at 37°C (200 µl/well).

Annex 13 (contd)

- v) Rinse four times with PBST.
- vi) Add 2% Triton X-100 to the virus suspension to be identified.
- vii) Dispense 100 µl/well of two- or four-step dilutions of the virus to be identified and of IHNV control virus, and a heterologous virus control (e.g. viral haemorrhagic septicaemia virus). Allow the samples to react with the coated antibody to IHNV for 1 hour at 20°C.
- viii) Rinse four times with PBST.
- ix) Add to the wells either biotinylated polyclonal IHNV antiserum or MAb to N protein specific for a domain different from the one of the coating MAb and previously conjugated with biotin.
- x) Incubate for 1 hour at 37°C.
- xi) Rinse four times with PBST.
- xii) Add streptavidin-conjugated horseradish peroxidase to those wells that have received the biotin-conjugated antibody, and incubate for 1 hour at 20°C.
- xiii) Rinse four times with PBST. Add the substrate and chromogen. Stop the course of the test when positive controls react and read the results.
- xiv) Interpretation of the results is according to the optical absorbencies achieved by negative and positive controls and must follow the guidelines for each test, e.g. absorbency at 450 nm of positive control must be minimum 5–10 × A450 of negative control.

The above biotin–avidin-based ELISA version is given as an example. Other ELISA versions of demonstrated performance may be used instead.

4.10. Other serological methods

Not applicable

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Virus isolation in cell culture or real-time RT-PCR are the recommended tests for surveillance to demonstrate freedom from infection with IHNV.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status³

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

³ For example transboundary commodities.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with IHN shall be suspected if at least one of the following criteria is met:

- i) Positive result by real-time RT-PCR;
- ii) IHN-typical CPE ~~Cytopathic effect~~ in cell culture.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with IHN is considered to shall be confirmed if, in addition to the criteria in Section 6.1.1., positive results has been obtained on at least one animal from two test used in the following combination one or more of the following criteria is met:

- i) Positive result by real-time ~~RT-RT-PCR~~ followed by and detection of IHN in a tissue sample by a positive result from a conventional PCR targeting a non-overlapping region of the genome and amplicon sequencing;
- ii) CPE Isolation of virus in cell culture confirmed by identified as IHN by real-time RT-PCR, conventional PCR, IFAT, or Ag-ELISA, or by a neutralisation test and a positive result followed by and detection of IHN in a tissue sample by real-time RT-PCR;
- iii) CPE Isolation of virus in cell culture confirmed by identified as IHN by real-time RT-PCR, conventional PCR, IFAT, or Ag-ELISA, or by a neutralisation test and followed by and detection of IHN in a tissue sample by conventional PCR and amplicon sequencing;
- iv) Positive result by real-time RT-PCR followed by isolation of virus in cell culture confirmed by identified as IHN by real-time RT-PCR, conventional PCR, IFAT, Ag-ELISA, or by a neutralisation test and amplicon sequencing.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.2. Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with IHN shall be suspected if at least one of the following criteria is met:

- i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality;
- ii) Positive result by real-time RT-PCR;
- iii) IHN-typical CPE ~~Cytopathic effect~~ in cell culture.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with IHN is considered to shall be confirmed if, in addition to the criteria in Section 6.2.1., positive results has been obtained on at least one animal from two tests used in the following combination one or more of the following criteria is met:

- i) Positive result by real-time ~~RT-RT-PCR~~ followed by and detection of IHN in a tissue sample a positive result from a conventional PCR targeting a non-overlapping region of the genome and amplicon sequencing;

Annex 13 (contd)

- ii) CPE Isolation of virus in cell culture confirmed by identified as IHNV by real-time RT-PCR, conventional PCR, IFAT, or Ag-ELISA, or by a neutralisation test and a positive result followed by and detection of IHNV in a tissue sample by real-time RT-PCR;
- iii) CPE Isolation of virus in cell culture confirmed by identified as IHNV by real-time RT-PCR, conventional PCR, IFAT, or Ag-ELISA, or by a neutralisation test and followed by and detection of IHNV in a tissue sample by conventional PCR and amplicon sequencing;
- iv) Positive result by real-time RT-PCR followed by isolation of virus in cell culture confirmed by identified as IHNV by real-time RT-PCR, conventional PCR, IFAT, Ag-ELISA, or by a neutralisation test and amplicon sequencing.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with IHNV are provided in Table 6.3. This information can be used for the design of surveys for infection with IHNV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

Table 6.1. Diagnostic performance of tests recommended for surveillance or diagnosis

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (n)	DSp (n)	Reference test	Citation
Real-time RT-PCR	Diagnosis	Experimentally infected salmon	Kidney	Steelhead (<i>Oncorhynchus mykiss</i>)	100 (50)	100 (50)	Animals of known infection status	Purcell <i>et al.</i> , 2013
RT-PCR (single step)	Diagnosis	Experimentally infected salmon	Kidney	Steelhead (<i>Oncorhynchus mykiss</i>)	58 (50)	100 (50)	Animals of known infection status	Purcell <i>et al.</i> , 2013
Virus Isolation	Diagnosis	Experimentally infected salmon	Kidney	Steelhead (<i>Oncorhynchus mykiss</i>)	84 (50)	100 (50)	Animals of known infection status	Purcell <i>et al.</i> , 2013
		Field samples	Kidney and spleen	Atlantic salmon (<i>Salmo salar</i>)	80-86 (50)	100 (50)	Clinical signs – history	McClure <i>et al.</i> , 2008

DSe = diagnostic sensitivity; DSp = diagnostic specificity; *n* = number of samples used in the study; RT-LAMP = real-time loop mediated isothermal amplification. *Listed as suitable test

7. References

ALONSO M. & LEONG J. (2013). Licensed DNA vaccines against infectious hematopoietic necrosis virus (IHNV). *Recent Pat. DNA Gene Seq.*, **7**, 62–65.

AUINGER B.M., PFANDL K. & BOENIGK J. (2008). Improved methodology for identification of protists and microalgae from plankton samples preserved in Lugol's iodine solution: combining microscopic analysis with single-cell PCR. *Appl. Environ. Microbiol.*, **74**, 2505–2510.

BALMER B.F., POWERS R.L., ZHANG T.-H., LEE J., VIGANT F., LEE B., JUNG M.E., PURCELL M.K., SNEKVIK K. & AGUILAR H.C. (2017). Inhibition of an aquatic rhabdovirus demonstrates promise of a broad-spectrum antiviral for use in aquaculture. *J. Virol.*, **91** (4), e02181–02116.

Annex 13 (contd)

BARLIC-MAGANJA D., STRANCAR M., HOSTNIK P., JENCIC V. & GROM J. (2002). Comparison of the efficiency and sensitivity of virus isolation and molecular methods for routine diagnosis of infectious haematopoietic necrosis virus and infectious pancreatic necrosis virus. *J. Fish Dis.*, **25**, 73–80.

BARROSO R.M., WHEELER P.A., LAPATRA S.E., DREW R.E. & THORGAARD G.H. (2008). QTL for IHNV resistance and growth identified in a rainbow trout (*Oncorhynchus mykiss*) X Yellowstone cutthroat (*Oncorhynchus clarki bouvieri*) trout cross. *Aquaculture*, **277**, 156–163.

BATTS W.N. & WINTON J.R. (1989). Concentration of infectious hematopoietic necrosis virus from water samples by tangential flow filtration and polyethylene glycol precipitation. *Can. J. Fish. Aquat. Sci.*, **46**, 964–968.

BOOTLAND L.M. & LEONG J.C. (1999). Infectious hematopoietic necrosis virus. *In: Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections*, Woo P.T.K. & Bruno D.W., eds. CAB International, Oxon, UK, 57–121.

BOVO G., HÅSTEIN T., HILL B., LAPATRA S.E., MICHEL C., OLESEN N.J., SHCHELKUNOV I., STORSET A., WOLLFROM T. & MIDTLYING P.J. (2005). Hazard identification for vertical transfer of fish disease agents. Available at: <http://www.crl-fish.eu/upload/sites/crl-fish/reports/links/fisheggtrade%20wp1.pdf>

BURBANK D.R., FEHRINGER T.R. & CHIARAMONTE L.V. (2017). Comparison of selected nonlethal samples from adult steelhead for detection of infectious hematopoietic necrosis virus using cell culture. *J. Aquat. Anim. Health*, **29**, 67–73.

BURKE J. & MULCAHY D. (1983). Retention of infectious haematopoietic necrosis virus infectivity in fish tissue homogenates and fluids stored at three temperatures. *J. Fish Dis.*, **6**, 543–547.

CIESLAK M., WAHLI T., DISERENS N., HAENEN O.L.M. & SCHUTZE H. (2017). Phylogeny of the infectious hematopoietic necrosis virus in European aquaculture. *PloS one*, **12** (9), e0184490.

DIXON P., PALEY R., ALEGRIA-MORAN R. & OIDTMANN B. (2016). Epidemiological characteristics of infectious hematopoietic necrosis virus (IHNV): a review. *Vet. Res.*, **47**, 63.

DROLET B.S., CHIOU P.P., HEIDEL J. & LEONG J.A. (1995). Detection of truncated virus particles in a persistent RNA virus infection *in vivo*. *J. Virol.*, **69**, 2140–2147.

DROLET B.S., ROHOVEC J.S. & LEONG J.C. (1994). The route of entry and progression of infectious haematopoietic necrosis virus in *Oncorhynchus mykiss* (Walbaum): a sequential immunohistochemical study. *J. Fish Dis.*, **17**, 337–347.

EMMENEGGER E.J., MEYERS T.R., BURTON T.O. & KURATH G. (2000). Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. *Dis. Aquat. Org.*, **40**, 163–176.

ENZMANN P.J., CASTRIC J., BOVO G., THIERY R., FICHTNER D., SCHÜTZE H. & WAHLI T. (2010). Evolution of infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, in Europe over 20 years: implications for control. *Dis. Aquat. Org.*, **89**, 9–15.

ENZMANN P.J., KURATH G., FICHTNER D. & BERGMANN S.M. (2005). Infectious hematopoietic necrosis virus: Monophyletic origin of European IHNV isolates from North-American genogroup M. *Dis. Aquat. Org.*, **66**, 187–195.

FOOTT J.S., FREE D., McDOWELL T., ARKUSH K.D. & HEDRICK R.P. (2006). Infectious hematopoietic necrosis virus transmission and disease among juvenile Chinook salmon exposed in culture compared to environmentally relevant conditions. *San Franc. Estuary Watershed Sci.*, **4** (1).

GARVER K.A., CONWAY C.M., ELLIOTT D.G. & KURATH G. (2005). Analysis of DNA-vaccinated fish reveals viral antigen in muscle, kidney and thymus, and transient histopathologic changes. *Mar. Biotechnol.*, **7**, 540–553. <https://doi.org/10.1007/s10126-004-5129>

GARVER K.A., BATTS W.N. & KURATH G. (2006). Virulence comparisons of infectious hematopoietic necrosis virus (IHNV) U and M genogroups in sockeye salmon and rainbow trout. *J. Aquat. Anim. Health*, **18**, 232–243. doi: 10.1577/h05-038.

Annex 13 (contd)

GARVER K.A., MAHONY-GRANT A.M., STUCCHI D.J., RICHARD J., VAN WOENSEL C. & FOREMAN M.G.G. (2013). Estimation of parameters influencing waterborne transmission of Infectious Hematopoietic Necrosis Virus (IHNV) in Atlantic Salmon (*Salmo salar*). *PLoS One*, **8** (12), e82296. doi: 10.1371/journal.pone.0082296. pmid:24340016.

GARVER K.A. & WADE J. (2017). Characterization of Infectious Hematopoietic Necrosis Virus (IHNV). *Canadian Science Advisory Secretariat (CSAS)*, **073**.

GOSTING L.H. & GOULD R.W. (1981). Thermal inactivation of infectious hematopoietic necrosis and infectious pancreatic necrosis viruses. *Appl. Environ. Microbiol.*, **41**, 1081–1082.

HARMACHE A., LEBERRE M., DROINEAU S., GIOVANNINI M. & BREMONT M. (2006). Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *J. Virol.*, **80**, 3655–3659.

HASOBE M. & SANAYOSHI M. (1985). On the approach to the viral chemotherapy against infectious hematopoietic necrosis virus (IHNV) *in vitro* and *in vivo* on salmonid fishes. *Fish Pathol.*, **20**, 343–351.

HOFERER M., AKIMKIN V., SKRYPISKI J., SCHÜTZE H. & STING R. (2019). Improvement of a diagnostic procedure in surveillance of the listed fish diseases IHN and VHS. *J. Fish Dis.*, **42**, 559–572.

HOSTNIK P., BARLIC-MAGANJA D., STRANCAR M., JENCIC V., TOPLAK I. & GROM J. (2002). Influence of storage temperature on infectious hematopoietic necrosis virus detection by cell culture isolation and RT-PCR methods. *Dis. Aquat. Org.*, **52**, 179–184.

JAKOB E., BARKER D.S. & GARVER K.A. (2011). Vector potential of the salmon louse *Lepeophtheirus salmonis* in the transmission of infectious haematopoietic necrosis virus (IHNV). *Dis. Aquat. Org.*, **97**, 155–165.

JOHANSSON T., EINER-JENSEN K., BATTS W.N., AHRENS P., BJÖRKBLOM C., KURATH G., BJÖRKLUND H. & LORENZEN N. (2009). Genetic and serological typing of European infectious haematopoietic necrosis virus (IHNV) isolates. *Dis. Aquat. Org.*, **86**, 213–221.

KAMEI Y., YOSHIMIZU M., EZURA Y. & KIMURA T. (1987). Effects of estuarine and marine waters on the infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). *Bull. Fac. Fish. Hokkaido Uni.*, **38**, 271–285.

KELL A.M., WARGO A.R. & KURATH G. (2014). Viral fitness does not correlate with three genotype displacement events involving infectious hematopoietic necrosis virus. *Virology*, **464**, 146–155.

KIM C.H., DUMMER D.M., CHIOU P.P. & LEONG J.A. (1999). Truncated particles produced in fish surviving infectious hematopoietic necrosis virus infection: mediators of persistence? *J. Virol.*, **73**, 843–849.

KNUSEL R., BERGMANN S.M., EINER-JENSEN K., CASEY J., SEGNER H. & WAHLI T. (2007). Virus isolation vs RT-PCR: which method is more successful in detecting VHSV and IHNV in fish tissue sampled under field conditions? *J. Fish Dis.*, **30**, 559–568. doi: JFD842 [pii]

KOŁODZIEJEK J., SCHACHNER O., DURRWALD R., LATIF M. & NOWOTNY N. (2008). “Mid-G” region sequences of the glycoprotein gene of Austrian infectious hematopoietic necrosis virus isolates form two lineages within European isolates and are distinct from American and Asian lineages. *J. Clin. Microbiol.*, **46**, 22–30.

KURATH G., GARVER K.A., TROYER R.M., EMMENEGGER E.J., EINER-JENSEN K. & ANDERSEN E.D. (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. *J. Gen. Virol.*, **84**, 803–814.

LAPATRA S.E. (1998). Factors affecting pathogenicity of infectious hematopoietic necrosis virus (IHNV) for salmonid fish. *J. Aquat Anim Health*, **10**, 121–131.

LAPATRA S.E., ROHOVEC J.S. & FRYER J.L. (1989). Detection of infectious hematopoietic necrosis virus in fish mucus. *Fish Pathol.*, **24**, 197–202.

UNOFFICIAL VERSION

MCCLURE C., SAKSIDA S., KARREMAN G., CONSTANTINE J., ROBINSON J., TRAXLER G. & HAMMELL L. (2008). Evaluation of a reverse transcriptase polymerase chain reaction test and virus isolation on field samples collected for the diagnosis of infectious hematopoietic necrosis virus in cultured Atlantic salmon in British Columbia. *J. Aquat. Anim. Health*, **20**, 12–18.

MEYERS T.R., KORN D., BURTON T.M., GLASS K., FOLLETT J.E., THOMAS J.B., SULLIVAN J., STARKEY N., SHORT S. & LIPSON K. (2003). Infectious hematopoietic necrosis virus (IHNV) in Alaskan sockeye salmon culture from 1973 to 2000: annual virus prevalences and titers in broodstocks compared with juvenile losses. *J. Aquat. Anim. Health*, **15**, 21–30.

MORZUNOV S.P., WINTON J.R. & NICHOL S.T. (1995). The complete genome structure and phylogenetic relationship of infectious hematopoietic necrosis virus. *Virus Res.*, **38**, 175–192.

MULCAHY D., BURKE J., PASCHO R. & JENES C.K. (1982). Pathogenesis of infectious hematopoietic necrosis virus in spawning sockeye salmon (*Oncorhynchus nerka*). *Can. J. Fish. Aquat. Sci.*, **39**, 1144–1149. 10.1111/j.1365-2761.2007.00842.x.

MULCAHY D., JENES C.K. & PASCHO R. (1984). Appearance and quantification of infectious hematopoietic necrosis virus in female sockeye salmon (*Oncorhynchus nerka*) during their spawning migration. *Arch. Virol.*, **80**, 171–181.

MULCAHY D. & PASCHO R.J. (1985). Vertical transmission of infectious haematopoietic necrosis virus in sockeye salmon, *Oncorhynchus nerka* (Walbaum): isolation of virus from dead eggs and fry. *J. Fish Dis.*, **8**, 393–396.

MULEI I.R., NYAGA P.N., MBUTHIA P.G., WARUIRU R.M., XU C., EVENSEN Ø. & MUTOLOKI S. (2019). First detection and isolation of infectious haematopoietic necrosis virus from farmed rainbow trout in Nyeri County, Kenya. *J. Fish Dis.*, **42**, 751–758. doi: 10.1111/jfd.12979. Epub 2019 Feb 26.

MULLER A., SUTHERLAND B.J.G., KOOP B.F., JOHNSON S.C. & GARVER K.A. (2015). Infectious hematopoietic necrosis virus (IHNV) persistence in Sockeye Salmon: influence on brain transcriptome and subsequent response to the viral mimic poly(I:C). *BMC Genomics*, **16**, 1–19.

NISHIZAWA T., KINOSHITA S., KIM W.S., HIGASHI S. & YOSHIMIZU M. (2006). Nucleotide diversity of Japanese isolates of infectious hematopoietic necrosis virus (IHNV) based on the glycoprotein gene. *Dis. Aquat. Org.*, **71**, 267–272.

OGUT H. & RENO P. (2004). Effects of fish density on spread of infectious hematopoietic necrosis virus (IHNV) in rainbow trout, *Oncorhynchus mykiss*. *Israeli J. Aquaculture*, **56**, 218–225.

OOI E.L., VERJANA N., HARAGUCHIA I., OSHIMA T., KONDO H., HIRONO I, AOKI T., KIYONO H & YUKI Y. (2008). Innate immunomodulation with recombinant interferon-alpha enhances resistance of rainbow trout (*Oncorhynchus mykiss*) to infectious hematopoietic necrosis virus. *Dev. Comp. Immunol.*, **32**, 1211–1220.

PASCOLI F., BILO F., MARZANO F.N., BORGHEGAN F., MANCIN M., MANFRIN A. & TOFFAN A. (2015). Susceptibility of genotyped marble trout *Salmo marmoratus* (Cuvier, 1829) strains to experimental challenge with European viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV). *Aquaculture*, **435**, 152–156.

PHAM P.H., SOKEECHAND B.S., GARVER K.A., JONES G., LUMSDEN J.S. & BOLS N.C. (2018). Fish viruses stored in RNA later can remain infectious and even be temporarily protected from inactivation by heat or by tissue homogenates. *J. Virol. Methods*, **253**, 31–37.

PIETSCH J.P., AMEND D.F. & MILLER C.M. (1977). Survival of infectious hematopoietic necrosis virus held under various environmental conditions. *J. Fish. Res. Board Can.*, **34**, 1360–1364.

PURCELL M.K., LAPATRA S.E., WOODSON J.C., KURATH G. & WINTON J.R. (2010). Early viral replication and induced or constitutive immunity in rainbow trout families with differential resistance to Infectious hematopoietic necrosis virus (IHNV). *Fish Shellfish Immunol.*, **28**, 98–105.

Annex 13 (contd)

PURCELL M.K., THOMPSON R., GARVER K.A., HAWLEY L.M., BATTS W.N., SPRAGUE L., SAMPSON C. & WINTON J.R. (2013). Universal reverse-transcriptase real-time PCR for infectious hematopoietic necrosis virus (IHNV). *Dis. Aquat. Org.*, **106**, 103–115.

SAKSIDA S.M. (2006). Infectious haematopoietic necrosis epidemic (2001 to 2003) in farmed Atlantic salmon *Salmo salar* in British Columbia. *Dis. Aquat. Org.*, **72**, 213–223.

SALONIUS K., SIMARD N., HARLAND R. & ULMER J.B. (2007). The road to licensure of a DNA vaccine. *Curr. Opin. Invest. Drugs*, **8**, 635–641.

ST-HILAIRE S., RIBBLE C., TRAXLER G., DAVIES T. & KENT M.L. (2001). Evidence for a carrier state of infectious hematopoietic necrosis virus in chinook salmon *Oncorhynchus tshawytscha*. *Dis. Aquat. Org.*, **46**, 173–179.

TRAXLER G.S. (1986). An epizootic of infectious hematopoietic necrosis in 2-year-old kokanee, *Oncorhynchus nerka* (Walbaum) at Lake Cowichan, British Columbia. *J. Fish Dis.*, **9**, 545–549.

TRAXLER G.S., ROOME J.R., LAUDA K.A. & LAPATRA S. (1997). Appearance of infectious hematopoietic necrosis virus (IHNV) and neutralizing antibodies in sockeye salmon *Oncorhynchus nerka* during their migration and maturation period. *Dis. Aquat. Org.*, **28**, 31–38.

WINTON J.R. (1991). Recent advances in the detection and control of infectious hematopoietic necrosis virus (IHNV) in aquaculture. *Ann. Rev. Fish Dis.*, **1**, 83–93.

WINTON J.R. & EINER-JENSEN K. (2002). Molecular diagnosis of infectious hematopoietic necrosis and viral hemorrhagic septicemia. *In: Molecular Diagnosis of Salmonid Diseases*, Cunningham C.O., ed. Kluwer, Dordrecht, The Netherlands, pp. 49–79.

WOLF K. (1988). Infectious hematopoietic necrosis. *In: Fish Viruses and Fish Viral Diseases*. Cornell University Press, Ithaca, New York, USA, pp. 83–114.

YAMAMOTO T., BATTS W.N., ARAKAWA C.K. & WINTON J.R. (1990). Multiplication of infectious hematopoietic necrosis virus in rainbow trout following immersion infection: Whole-body assay and immunohistochemistry. *J. Aquat. Anim. Health*, **2**, 271–280.

YOSHIMIZU M., YOSHINAKA T., HATORI S. & KASAI H. (2005). Survivability of fish pathogenic viruses in environmental water, and inactivation of fish viruses. *Bull. Fish Res. Agency*, **2**, 47–54.

*
* *

NB: There are OIE Reference Laboratories for Infection with infectious haematopoietic necrosis virus (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: <http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratories for any further information on infection with viral haemorrhagic septicaemia virus

NB: FIRST ADOPTED IN 1995 AS INFECTIOUS HAEMATOPOIETIC NECROSIS;
MOST RECENT UPDATES ADOPTED IN 2019.

CHAPTER 2.3.10.

INFECTION WITH VIRAL HAEMORRHAGIC SEPTICAEMIA VIRUS

EU comment

The EU thanks the OIE and in general supports the proposed changes to this chapter.

One comment is inserted in the text below.

1. Scope

Infection with viral haemorrhagic septicaemia virus (VHSV) means infection with the pathogenic agent viral haemorrhagic septicaemia virus of the Genus *Novirhabdovirus* and Family *Rhabdoviridae*.

2. Disease information**2.1. Agent factors****2.1.1. Aetiological agent**

VHSV is a bullet-shaped particle, approximately 70 nm in diameter and 180 nm in length, that contains a negative-sense, single-stranded RNA genome of approximately 11,000 nucleotides, and possesses an envelope that contains the membrane glycoprotein, which is the neutralising surface antigen. The genome encodes six proteins: a nucleoprotein N; a phosphoprotein P (formerly designated M1); a matrix protein M (formerly designated M2); a glycoprotein G; a non-virion protein NV and a polymerase L (Walker *et al.*, 2000).

G-gene nucleotide sequences have been used to classify VHSV isolates into four major genotypes (I, II, III and IV) and nine subtypes (Ia–Ie and IVa–IVd) with almost distinct geographical distributions (Einer-Jensen *et al.*, 2004; Elsayed *et al.*, 2006). The host range and the pathogenicity appear, at least to some extent, to be linked to the genotype of VHSV.

i) Genotype Ia

Almost all VHSV isolates causing outbreaks in European rainbow trout (*Oncorhynchus mykiss*) farms cluster in sub-lineage Ia, of which isolates have been reported from most continental European countries (Einer-Jensen *et al.*, 2004; Kahns *et al.*, 2012; Snow *et al.*, 2004; Toplak *et al.*, 2010). However, genotype Ia isolates have also been detected in other species **in Europe** such as brown trout (*Salmo trutta*), pike (*Esox lucius*) and grayling (*Thymallus thymallus*) (de Kinkelin & Le Berre, 1977; Jonstrup *et al.*, 2009). Genotype Ia isolates have generally caused outbreaks in **European** freshwater farms, but isolates have also been obtained from rainbow trout in seawater net pens and turbot (*Scophthalmus maximus* **syn. Psetta maxima**) (Schlotfeldt *et al.*, 1991; Snow *et al.*, 2004). Genotype Ia can be further subdivided into two major subpopulations, Ia-1 and Ia-2, with a distinct geographic distribution within Europe (Kahns *et al.*, 2012).

ii) Genotype Ib

The isolates included in this genotype ~~Viruses~~ have been **isolated-obtained** from fish in the marine environment in the Baltic Sea, Kattegat, Skagerrak, the North Sea and the English Channel (Einer-Jensen *et al.*, 2004; Skall *et al.*, 2005**b**; Snow *et al.*, 2004) and as far north as latitude 70°N close to Nordkapp in Norway (Sandlund *et al.*, 2014). A single case was observed in Japan (Nishizawa *et al.*, 2002). None of the isolations from wild fish has been associated with clinical disease outbreaks (Johansen *et al.*, 2013). Genotype Ib has been associated with evidence of transfer between wild fish and farmed rainbow trout in only two cases in pen-reared rainbow trout in Sweden in 1998 and 2000 (Nordblom, 1998; Nordblom & Norell, 2000; Skall *et al.*, 2005**a**).

iii) Genotype Ic

This genotype **consists of** ~~is~~ a smaller group **consisting** of Danish **isolates** from farmed rainbow trout **isolates from earlier dates**. Isolates of this genotype have also been **identified-detected** in Germany and Austria (Jonstrup *et al.*, 2009).

iv) Genotype Id

~~This group~~ ~~The isolates included in this genotype~~ consists of some old Scandinavian isolates from the 1960s until the first VHS outbreaks of infection with VHSV occurred in Finland in sea-reared rainbow trout in 2000. ~~These outbreaks occurred in at two different areas where and all of the isolates sampled were proved to clustered~~ in the Id genotype group. In infection trials, it was demonstrated that the isolates were pathogenic to rainbow trout, but less virulent than most Ia isolates (Raja-Halli *et al.*, 2006).

v) Genotype Ie

These isolates ~~included in this genotype~~ have been obtained from both freshwater and marine (the Black Sea) environments in Georgia and Turkey. Isolations were from both farmed and wild turbot (Jonstrup *et al.*, 2009; Kalayci *et al.*, 2006; Nishizawa *et al.*, 2006) and from rainbow trout (Einer-Jensen *et al.*, 2004). VHSV Ie has also been ~~identified isolated from in~~ whiting (*Merlangius merlangus*) and sea bass (*Dicentrarchus labrax*) ~~from in~~ the Black Sea (Altuntas & Ogut, 2010).

vi) Genotype II

The ~~members isolates included in of this group genotype consist of have been primarily~~ detected in marine ~~isolates from~~ wild fish, ~~in particular especially from~~ Atlantic herring (*Clupea harengus*), ~~from in~~ the Baltic Sea, including the Gulf of Bothnia and the Gulf of Finland, (Gadd *et al.*, 2011; Snow *et al.*, 2004). ~~Genotype II isolates have also been detected~~ in lamprey (*Lampetra fluviatilis*) ~~caught in freshwater~~ from the rivers Kalajoki and Lestijoki, ~~which have~~ an outlet into the Gulf of Bothnia (Gadd *et al.*, 2010).

vii) Genotype III

These isolates ~~included in this genotype~~ originate from wild and farmed fish in the North Atlantic Sea from the Flemish Cap (Lopez-Vazquez *et al.*, 2006b) to the Norwegian coast (Dale *et al.*, 2009), the North Sea, around the British Isles, Skagerrak and Kattegat. ~~VHS outbreaks~~ ~~Outbreaks of infection with VHSV~~ in farmed turbot in the United Kingdom and Ireland in the 1990s were ~~attributed due to infection with~~ genotype III isolates, and in 2007 an outbreak in sea-reared rainbow trout at the Norwegian west coast was due to VHSV genotype III. ~~VHS Outbreaks of infection with VHSV~~ in five species of wrasse used as cleaner fish around the Shetland Islands were also due to this genotype (Munro *et al.*, 2015).

viii) Genotype IVa

~~The~~ isolates ~~included in this genotype have been detected originate in finfish~~ from both the east and west coasts of North America, as well as from the Asian countries of South Korea and Japan. Genotype IVa isolates in North America have caused severe epidemics in numerous wild marine species such as Pacific herring (*Clupea pallasii pallasii*) (Meyers & Winton, 1995), which can serve as a reservoir of virus to sympatric net-pen farmed Atlantic salmon (*Salmo salar*) (Garver *et al.*, 2013). In Asia, genotype IVa isolates have caused disease outbreaks in ~~olive flounder bastard halibut~~ (*Paralichthys olivaceus*) (Ogut & Altuntas, 2014).

ix) Genotype IVb

~~The~~ isolates ~~included in this genotype have been detected originate in finfish originate~~ from the North America Laurentian Great Lakes region (Gagne *et al.*, 2007; Thompson *et al.*, 2011; Winton *et al.*, 2008) ~~and where they have caused die-offs events in numerous fish species, and have been detected in a micro-invertebrate (Diporeia spp.)~~ (Faisal & Winters, 2011).

x) Genotype IVc

~~The~~ isolates ~~included in this genotype originate have been detected from finfish~~ from the estuarine waters of New Brunswick and Nova Scotia, Canada (Gagne *et al.*, 2007; Pierce & Stepien, 2012; Stepien *et al.*, 2015).

xi) Genotype IVd

~~The~~ isolates ~~included in this genotype originate have been detected in from~~ Iceland where they were identified in wild and farmed lumpfish (*Cyclopterus lumpus*) (Gudmundsdottir *et al.*, 2019).

2.1.2. Survival and stability in processed or stored samples

VHSV survival in host tissue is dependent on the conditions for storage. VHSV remains infectious for long time periods while stored frozen in fish tissue. However, VHSV-infected fish at commercial freezing temperatures (-20°C) had a 90% reduction in viral titre after the tissue was thawed (Arkush *et al.*, 2006). VHSV is sensitive to enzymatic degradation, environments with high bacterial load and high temperatures (above 28°C). Fresh (unfrozen) muscle tissue from VHSV-infected rainbow trout could transmit ~~infection with~~ VHSV to naive fish (Oidtmann *et al.*, 2011). VHSV is also tolerant of high salt concentrations such as in brine-treated fish (Skall *et al.*, 2015) or while stored in concentrated ammonium sulphate solution (Pham *et al.*, 2018). For optimal retention of VHSV in fish tissue, the

sample should be placed in transport medium with antibiotics and kept on ice without freezing and processed within 24 hours after sampling.

2.1.3. Survival and stability outside the host

VHSV survival outside the host is dependent on the physico-chemical conditions of the aqueous medium (Ahne, 1982) and on temperature: the virus survives for longer periods at 4°C compared with 20°C (Parry & Dixon, 1997).

VHSV is significantly more stable in freshwater than saltwater. The virus has been documented to persist in freshwater for 28–35 days at 4°C (Parry & Dixon, 1997) and has been found to be infective for 1 year at 4°C in filtered freshwater (Hawley & Garver, 2008). In raw freshwater at 15°C, the 99.9% inactivation time was 13 days, but in seawater the virus was inactivated within 4 days (Hawley & Garver, 2008). In another study using seawater at 15°C, the infectivity of the virus was reduced by 50% after 10 hours, but could still be recovered after 40 hours (Kocan *et al.*, 2001). There appears to be no consistent correlation between the origin and stability of the virus isolates: freshwater isolates are not always the most stable in freshwater and seawater isolates are not consistently more stable in seawater (Hawley & Garver, 2008).

The virus remains stable for a longer time if sterile organic materials are added to the water, such as ovarian fluids or blood products, such as bovine serum (Kocan *et al.*, 2001). When the sea water was sterilised by autoclaving, or when passed through a 0.22 µm membrane, virus survival was prolonged significantly (60 days at 15°C and 32 days at 20°C), suggesting the bacterial load in the water is an important factor of viral decay.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with VHSV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include:

Family	Scientific name	Common name	Genotype
Ammodytidae	<i>Ammodytes hexapterus</i>	Pacific sand lance	IVa
Aralichthyidae	<i>Paralichthys olivaceus</i>	Bastard halibut	IVa
Carangidae	<i>Trachurus mediterraneus</i>	Mediterranean horse mackerel	Ie
Centrarchidae	<i>Ambloplites rupestris</i>	Rock bass	IVb
	<i>Lepomis gibbosus</i>	Pumpkinseed	IVb
	<i>Lepomis macrochirus</i>	Bluegill	IV, IVb
	<i>Micropterus dolomieu</i>	Smallmouth bass	IVb
	<i>Micropterus salmoides</i>	Largemouth bass	IVb
	<i>Pomoxis nigromaculatus</i>	Black crappie	IVb
Clupeidae	<i>Alosa immaculata</i>	Pontic shad	Ie
	<i>Sardina pilchardus</i>	Pilchard	ND
	<i>Clupea harengus</i>	Atlantic herring	Ib, III
	<i>Clupea pallasii pallasii</i>	Pacific herring	IVa
	<i>Dorosoma cepedianum</i>	American gizzard shad	IVb
	<i>Sardinops sagax</i>	South American pilchard	IVa
	<i>Sprattus sprattus</i>	European sprat	Ib
Cyclopteridae	<i>Cyclopterus lumpus</i>	Lumpfish	IVd
Cyprinidae	<i>Danio rerio</i>	Zebra fish	IVa
	<i>Notropis hudsonius</i>	Spottail shiner	IVb
	<i>Notropis atherinoides</i>	Emerald shiner	IVb
	<i>Pimephales notatus</i>	Bluntnose minnow	IVb
	<i>Pimephales promelas</i>	Fathead minnow	IVb
Embiotocidae	<i>Cymatogaster aggregata</i>	Shiner perch	IVa
Engraulidae	<i>Engraulis encrasicolus</i>	European anchovy	Ie
Esocidae	<i>Esox lucius</i>	Northern pike	IVb
	<i>Esox masquinongy</i>	Muskellunge	IVb
Fundulidae	<i>Fundulus heteroclitus</i>	Mummichog	IVc
Gadidae	<i>Gadus macrocephalus</i>	Pacific cod	IVa
	<i>Gadus morhua</i>	Atlantic cod	Ib, III
	<i>Merlangius merlangus</i>	Whiting	Ie
	<i>Micromesistius poutassou</i>	Blue whiting	Ib, III
	<i>Trisopterus esmarkii</i>	Norway pout	Ib, III

Annex 14 (contd)

Gasterosteidae	<i>Gasterosteus aculeatus</i>	Three-spine stickleback	IVc
Gobiidae	<i>Neogobius melanostomus</i>	Round goby	IVb
	<i>Pomatoschistus minutus</i>	Sand goby	Ib
Ictaluridae	<i>Ictalurus nebulosus</i>	Brown bullhead	IVb
Labridae	<i>Centrolabrus exoletus</i>	Rock cook wrasse	III
	<i>Ctenolabrus rupestris</i>	Goldsinny wrasse	III
	<i>Labrus bergylta</i>	Ballan wrasse	III
	<i>Labrus mixtus</i>	Cuckoo wrasse	III
	<i>Symphodus melops</i>	Corkwing wrasse	III
Lotidae	<i>Gaidropsarus vulgaris</i>	Three-bearded rockling	Ie
Moronidae	<i>Morone americana</i>	White perch	IVb
	<i>Morone chrysops</i>	White bass	IVb
	<i>Morone saxatilis</i>	Striped bass	IVb, IVc
Mullidae	<i>Mullus barbatus</i>	Red mullet	Ie
Osmeridae	<i>Thaleichthys pacificus</i>	Eulachon	IVa
Percidae	<i>Sander vitreus</i>	Walleye	IVb
	<i>Perca flavescens</i>	Yellow perch	IVb
Petromyzontidae	<i>Lampetra fluviatilis</i>	River lamprey	II
Pleuronectidae	<i>Limanda limanda</i>	Common dab	Ib
	<i>Platichthys flesus</i>	European flounder	Ib
	<i>Pleuronectes platessus</i>	European plaice	III
Rajidae	<i>Raja clavata</i>	Thornback ray	Ie
Salmonidae	<i>Coregonus artedii</i>	Lake cisco	IVb
	<i>Coregonus clupeaformis</i>	Lake whitefish	IVb
	<i>Coregonus lavaretus</i>	Common whitefish	Ia
	<i>Oncorhynchus kisutch</i>	Coho salmon	IVa
	<i>Oncorhynchus mykiss</i>	Rainbow trout	Ia-e, III, IVb
	<i>Oncorhynchus mykiss</i> X <i>Oncorhynchus kisutch</i> hybrids	Rainbow trout X coho salmon hybrids	Ia
	<i>Oncorhynchus tshawytscha</i>	Chinook salmon	IVa, IVb
	<i>Salmo marmoratus</i>	Marble trout	Ia
	<i>Salmo salar</i>	Atlantic salmon	Ia, Ib, II, III, IVa
	<i>Salmo trutta</i>	Brown trout	Ia, Ib
	<i>Salvelinus namaycush</i>	Lake trout	Ia, IVa, IVb
<i>Thymallus thymallus</i>	Grayling	I	
Scophthalmidae	<i>Scophthalmus maxima</i>	Turbot	Ib, III
Sciaenidae	<i>Aplodinotus grunniens</i>	Freshwater drum	IVb
Scombridae	<i>Scomber japonicus</i>	Pacific chub mackerel	IVa
Soleidae	<i>Solea senegalensis</i>	Senegalese sole	III
Uranoscopidae	<i>Uranoscopus scaber</i>	Atlantic stargazer	Ie

ND: Not determined.

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence to fulfil the criteria for listing as susceptible to infection with VHSV according to Chapter 1.5 of the *Aquatic Code* include:

Family	Scientific name	Common name	Genotype
Adrianichthyidae	<i>Oryzias latipes</i>	Japanese rice fish	IVb
	<i>Oryzias dancena</i>	Marine medaka	IVa
Ammodytidae	<i>Ammodytes personatus</i>	Sandeel	Ib
Anguillidae	<i>Anguilla anguilla</i>	European eel	III
Argentinidae	<i>Argentina sphyraena</i>	Lesser Argentine	Ib
Belonidae	<i>Belone belone</i>	Garfish	Ie
Carangidae	<i>Seriola dumerili</i>	Greater amberjack	IVa
Catostomidae	<i>Catostomus commersonii</i>	White sucker	IVb
	<i>Moxostoma anisurum</i>	Silver redhorse	IVb
	<i>Moxostoma macrolepidotum</i>	Shorthead redhorse	IVb
Centrarchidae	<i>Pomoxi annularis</i>	White crappie	IVb

Clupeidae	<i>Alosa pseudoharengus</i>	Alewife	IVb
	<u><i>Clupea harengus</i>*</u>	<u>Atlantic herring*</u>	<u>IVa</u>
Cottidae	<i>Cottus pollux</i>	Japanese fluvial sculpin	IVb
Cyprinidae	<i>Semotilus corporalis</i>	Fallfish	IVb
	<i>Notemigonus crysoleucas</i>	Golden shiner	IVb
Esocidae	<i>Esox lucius</i> X <i>E. masquinongy</i> hybrids	Tiger muskellunge (<i>Esox masquinongy</i> X <i>E. lucius</i> or <i>E. lucius</i> X <i>E. masquinongy</i>)	IVb
Fundulidae	<i>Fundulus diaphanus</i>	Banded killifish	IVb
Gadidae	<i>Gadiculus argenteus</i>	Silvery pout	Ib
	<i>Melanogrammus aeglefinus</i>	Haddock	III
	<i>Theragra chalcogramma</i>	Alaska pollock	IVa
	<i>Trisopterus minutus</i>	Poor cod	III
Ictaluridae	<i>Ictalurus punctatus</i>	Channel catfish	IVb
Liparidae	<i>Liparis tessellatus</i>	Cubed snailfish	IV
Lotidae	<i>Lota lota</i>	Burbot	IVb
	<i>Enchelyopus cimbrius</i>	Fourbeard rockling	Ib
Merlucciidae	<i>Merluccius productus</i>	North Pacific hake	IVa
Moronidae	<i>Dicentrarchus labrax</i>	European sea bass	Ia
Mugilidae	<i>Mugil cephalus</i>	Flathead grey mullet	IV
Ophidiidae	<i>Hoplobrotula armata</i>	Armoured cusk	IV
Osmeridae	<i>Hypomesus pretiosus</i>	Surf smelt	ND
Oxudercidae	<i>Rhinogobius</i> sp. (undescribed species)	Yoshinobori	IVb
Percopsidae	<i>Percopsis omiscomaycus</i>	Trout perch	IVb
Pleuronectidae	<i>Glyptocephalus stelleri</i>	Blackfin flounder	IVa
	<i>Hippoglossus hippoglossus</i>	Atlantic halibut	III
	<i>Reinhardtius hippoglossoides</i>	Greenland halibut	III
Salmonidae	<i>Oncorhynchus mykiss</i> X <i>Salvelinus alpinus</i> hybrids	Rainbow trout X Arctic charr hybrids	Ia
	<i>Oncorhynchus mykiss</i> X <i>Salvelinus namaycush</i> hybrids	Rainbow trout X lake trout hybrids	<u>Ia</u>
	<i>Oncorhynchus mykiss</i> X <i>Salmo trutta</i> hybrids	Rainbow trout X brown trout hybrids	Ia
	<i>Salvelinus alpinus</i>	Arctic charr	Ia
	<i>Salvelinus fontinalis</i>	Brook trout	Ie
Sciaenidae	<i>Larimichthys polyactis</i>	Yellow croaker	IV
Scorpaenidae	<i>Scorpaena porcus</i>	Black scorpionfish	Ie
	<i>Scorpaena izensis</i>	Izu scorpionfish	IV
Scyliorhinidae	<i>Scyliorhinus torazame</i>	Claudy catshark	IV
Stromateidae	<i>Pampus argenteus</i>	Silver pomfret	IV
Trichiuridae	<i>Trichiurus lepturus</i>	Largehead hairtail	IV
Triglidae	<i>Eutrigla gurnardus</i>	Gray gurnard	III

ND: Not determined.

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following organisms, but an active infection has not been demonstrated: Sablefish (*Anoplopoma fimbria*).

2.2.3. Non-susceptible species

None known.

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

Rainbow trout is the most susceptible species to VHSV infection with genotype Ia. For VHSV genotypes Ib, II and III, shoaling wild-living species such as Atlantic herring and European sprat (*Sprattus sprattus*) are likely to be the natural hosts, while for genotype IVa, Pacific herring is the natural host. VHSV genotype III has caused disease in farmed turbot and wrasse and genotype IVa in farmed Atlantic salmon, turbot, and olive flounder-bastard halibut.

Annex 14 (contd)

Infection with VHSV may cause disease and mortality in all life stages of susceptible fish. VHSV does not infect fish eggs (Munro & Gregory, 2010).

In surveys of wild marine fish, VHSV has been isolated from most year classes. Few fry have been tested however, as they are usually not caught during the surveys. The highest prevalence of virus in sampled wild populations was found in shoaling fish, such as Atlantic herring, European sprat and Norway pout (*Sprattus sprattus*) (Skall *et al.*, 2005a).

2.2.5. Distribution of the pathogen in the host

In fish showing clinical signs, the virus is abundant in all tissues including gill, skin and muscles (Sandlund *et al.*, 2014). Target organs are anterior kidney, heart and spleen, as these are the sites in which virus is most abundant. In chronic stages, virus titres can become high in the brain (Smail & Snow, 2011; Wolf, 1988).

2.2.6. Aquatic animal reservoirs of infection

Some survivors of epizootics will become long-term carriers of the virus. Pacific herring surviving infection with VHSV genotype IVa have transmitted disease to naïve cohabitants (Gross *et al.*, 2019). Almost all isolations of VHSV genotype Ib, II and III from free-living fish species are from individuals with no clinical signs of infection with VHSV and with low virus titres (Skall *et al.*, 2005a).

2.2.7. Vectors

VHSV has been isolated from common snapping turtle (*Chelra serpentina*), leech (*Myzobdella lugubris*), northern map turtle (*Graptemys geographicas*) and water flea (*Moina macrocopa*) and these species are considered may be potential to be vectors for transmission of VHSV rather than true susceptible species (Faisal & Schultz, 2009; Goodwin & Merry, 2011; Ito & Olesen, 2017). VHSV has also been isolated from the amphipods *Hyalella* spp. and *Diporeia* spp., suggesting that benthic macroinvertebrates may be vectors for VHSV IVb in endemically affected systems. In contrast VHSV was not detected in mussels or sediments in the same water environment (Faisal & Winters 2011; Throckmorton *et al.*, 2017). VHSV has also been isolated from leech, *Myzobdella lugubris*, in the Great Lakes but whether the leech or amphipods can transmit VHSV from one fish to another is unknown (Faisal & Schulz, 2009; Faisal & Winters, 2011).

Piscivorous birds may act as VHSV vectors by carrying the virus, for example, on their feet (Olesen & Jorgensen, 1982), or through regurgitation of infected fish (Peters & Neukirch, 1986).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Mortality varies, depending on many environmental and physiological conditions, most of which have not been fully determined. The disease is, in general, a cool or cold water disease with highest mortality at temperatures around 9–12°C. Small rainbow trout fry (0.3–3 g) are most susceptible to genotype Ia with mortalities close to 100%, but all sizes of rainbow trout can be affected with mortalities ranging from 5 to 90% (Skall *et al.*, 2004). Immersion infection trials also induced up to 100% mortality in Pacific herring when challenged with genotype IVa (Hershberger *et al.*, 2010). Mortality in free living fish also varies from no observable deaths to severe die-offs. The prevalence of VHSV genotype Ib, II and III varies from 0 to 16.7% in Northern European waters (Skall *et al.*, 2005b).

2.3.2. Clinical signs, including behavioural changes

The occurrence of the following clinical signs is characteristic of infection with VHSV: rapid onset of mortality, lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins or in the gills, eyes or skin, abnormal swimming such as flashing and spiralling, and a distended abdomen due to oedema in the peritoneal cavity. In rainbow trout, the clinical appearance is typically lethargic dark fish with exophthalmia at the pond shores and the outlet. Characteristically, diseased fish will not attempt to escape when netted.

Some genotypes have specific predominant clinical signs of infection with VHSV. Skin lesions in cod and herring from the Pacific and Atlantic Oceans (including the North Sea), and in haddock from the North Sea, have been described frequently (Jensen & Larsen, 1979; Meyers *et al.*, 1992; Meyers & Winton, 1995; Smail, 2000; Vestergaard Jorgensen & Olesen, 1987). In farmed Japanese flounder, an 'anaemic' form (pale gills) of infection with VHSV has also been described (Isshiki *et al.*, 2001).

EU comment

The Commission has not agreed with a request to add 'olive flounder' as a common name for bastard halibut (*Paralichthys olivaceus*). However, the paragraph above still uses the common name 'Japanese flounder'. For consistency we would like to suggest that 'bastard halibut' is used instead of 'Japanese flounder'.

2.3.3 Gross pathology

Gross pathology includes generalised petechial haemorrhaging in the skin, muscle tissue (especially in dorsal muscles) and internal organs. It is important to examine the dorsal musculature for the presence of petechial bleeding, which is a very common sign of infection with VHSV. The kidney is dark red in the acute phase and can demonstrate severe necrosis in moribund fish. The spleen is moderately swollen. The liver is often pale and mottled. The gastrointestinal tract, especially the hind gut, is pale and devoid of food.

2.3.4. Modes of transmission and life cycle

Transmission primarily occurs horizontally through water, with excretion of virus in the urine, and directly from the skin (Smail & Snow, 2011). Oral transmission was also demonstrated indicating that preying on infected fish and vectors may transfer the disease (Schonherz *et al.* 2012).

Experimentally it has been demonstrated that feeding fresh (unfrozen) muscle tissue from VHSV-infected rainbow trout can transmit VHSV to naïve fish (Oidtmann *et al.*, 2011).

There are no indications or evidence of true vertical transmission of VHSV (Bovo *et al.*, 2005a; Munro & Gregory, 2010).

2.3.5. Environmental and management factors

Disease generally occurs at temperatures between 4°C and 14°C. At water temperatures between 15°C and 18°C, the disease generally takes a short course with low levels of mortality.

Low water temperatures (1–5°C) generally result in an extended disease course with low daily mortality but high accumulated mortality. Outbreaks of infection with VHSV occur during all seasons but are most common in spring when water temperatures are rising or fluctuating.

Field observations and experimental studies suggest that warmer water temperatures greatly reduce or inhibit transmission. Natural outbreaks of infection with VHSV are not observed at water temperatures greater than 18°C. In challenge trials, fish exposed to VHSV and reared at temperatures below 15°C displayed high mortality whereas those infected and reared at 20°C did not (Arkush *et al.*, 2006; Castric & de Kinkelin, 1984). For more detailed reviews, see Wolf (1988) and Smail & Snow (2011).

2.3.6. Geographical distribution

Until the late 1980s, VHSV was considered to be restricted to farmed rainbow trout in continental Europe, with the occasional isolation from a restricted number of other freshwater fish species (e.g. brown trout, pike [Meier & Jorgensen, 1980; Schlotfeldt & Ahne, 1988]). With the detection and isolation of VHSV from Pacific salmon off the Pacific North American coast in the late 1980s, subsequent studies have demonstrated that infection with VHSV occurs in numerous farmed and wild fish species along the Pacific and Atlantic North American coast (Skall *et al.*, 2005), in the Great Lakes area of North America (Thompson *et al.*, 2011), the seas around the UK (Skall *et al.*, 2005), the Baltic Sea, Skagerrak and Kattegat (Skall *et al.*, 2005), in the waters around Japan (Skall *et al.*, 2005), and in the Black Sea area, with the distinct genotype 1c (Nishizawa *et al.*, 2006).

Infection with VHSV in farmed rainbow trout has been reported from countries in Europe, North America and North Asia. Some countries in these regions have declared freedom from infection with VHSV, almost all European and Middle East countries and from China (People's Rep. of) and Russia. However,

a number of countries in Europe, such as Denmark, Ireland, Norway, Sweden and UK, are officially declared free of infection with VHSV. Infection with VHSV The disease has never been reported from the Southern Hemisphere.

For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Although research on vaccine development for VHSV has been ongoing for more than four decades, the only a-commercial vaccine is not yet available is against VHSV genotype IVa for bastard halibut in Korea. Candidate vaccines have included killed vaccines, attenuated live vaccines, a recombinant vaccine in prokaryotic and eukaryotic expression systems, and DNA-based vaccines. For a review see Lorenzen & LaPatra (2005). No vaccines currently affect the sensitivity and specificity of infection with VHSV diagnostics.

2.4.2. Chemotherapy including blocking agents

No therapies are currently available.

2.4.3. Immunostimulation

Several immunostimulants, such as yeast-derived beta-glucans, IL-1 β -derived peptides, and probiotics have been assessed for enhancing protection against infection with VHSV (Peddie *et al.*, 2003). Several researchers report positive effects, but no immunostimulant directed specifically at enhanced resistance to infection with VHSV is available. Furthermore, it remains unknown as to whether their use can affect sensitivity and specificity of infection with VHSV assays/diagnostics.

2.4.4. Breeding resistant strains

Additive genetic variation in rainbow trout has been detected for resistance to infection with VHSV has been demonstrated (Dorson *et al.*, 1995; Henryon *et al.*, 2002a; 2002b). In a study by Henryon *et al.* (2005), the heritability of resistance to VHS was 0.11 for time to death on a logarithmic timescale. Identification of a major quantitative trait loci (QTL) for VHSV resistance in rainbow trout may pave the way for genetic selection for VHSV resistant fish (Verrier *et al.*, 2013), however, no resistant rainbow trout strains are yet-commercially available.

2.4.5. Inactivation methods

VHSV is sensitive to a number of common disinfectants (e.g. UV, chlorine, iodophore, sodium hypochlorite), to temperatures above 30°C, to bacterial degradation in sediments and enzymatic activity in decomposing fish. For a review see Bovo *et al.*, 2005b.

2.4.6. Disinfection of eggs and larvae

Disinfection of newly fertilised or eyed and green eggs is an efficient and cost-effective preventative measure for stopping the spread of the disease in salmonids (for the recommended protocol see Chapter 4.4. of the *Aquatic Code*).

2.4.7. General husbandry

Poor water quality, high fish density, high feeding rate, infection with other diseases such as proliferative kidney disease, ichthyophthiriasis, bacterial kidney disease, etc. can influence the course and severity of infection with VHSV. In general, an increase in temperature, restricted feeding, reduced fish density and restricted handling may reduce mortality. In endemically infected farms, stocking with naïve fry is usually done at as high when the water temperatures is at near maximum levels as possible.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when the water temperature is below 14°C or whenever the water temperature is likely to reach its lowest annual point. All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Particular attention should be paid to the water outlet area where weak fish tend to accumulate due to the water current.

Fish to be sampled are selected as follows:

- i) For genotype I, in farms where rainbow trout are present, **only** fish of that species should be selected for sampling. If rainbow trout are not present, the sample should be obtained from fish of all other VHSV-susceptible species present (as listed in Tables 2.1) **and or from species with incomplete evidence for susceptibility (as listed in Table 2.2)**. However, the species should be proportionally represented in the sample. For other genotypes (II, III, and IV), species of known susceptibility to the genotype in question should be sampled.
- ii) Susceptible species should be sampled following risk-based criteria for targeted selection of populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or introduction of stocks of unknown risk status).
- iii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.

3.2. Selection of organs or tissues

The optimal tissues **material to be examined is are** spleen, anterior kidney, heart and encephalon. **When sampling broodstock, in some cases, ovarian fluid and milt can be taken must be examined.**

In case of small fry, whole fish less than 4 cm long can be minced with sterile scissors or a scalpel after removal of the body behind the **anal pore-gut opening**. If a sample consists of whole fish with a body length between 4 cm and 6 cm, the viscera including kidney should be collected. For larger size fish, kidney, spleen, heart and encephalon, and ovarian fluid from brood fish at the time of spawning should be the tissues to be sampled.

3.3. Samples or tissues not suitable for pathogen detection

VHSV is very sensitive to enzymatic degradation, therefore, sampling tissues with high enzymatic activities, such as viscera and liver, or large numbers of contaminating bacteria, such as the intestine or skin, should be avoided. Preservatives and fixatives, such as RNAlater and formaldehyde can be toxic to tissue culture cells such as epithelioma papulosum cyprini (EPC) and fathead minnow (FHM), and can impact molecular detection methods (Auinger *et al.*, 2008; Pham *et al.*, 2018).

3.4. Non-lethal sampling

Fin and gill biopsies were shown to be effective nonlethal samples for detection of VHSV genotype IVb (Cornwell *et al.*, 2013) **in clinically diseased fish** and nested reverse-transcription polymerase chain reaction (RT-PCR) on blood samples from infected fish was **also** shown to be **effective-efficient** for VHSV detection (Lopez-Vazquez *et al.*, 2006a). In the case of brood fish, ovarian fluid and milt can be used for testing as **an** alternative to lethal testing. However, **no non-lethal samplings methods have not** been fully validated for detection of all VHSV genotypes **and are therefore not prescribed in this chapter.**

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

3.5.1. Samples for pathogen isolation

The success of pathogen isolation and results of bioassay depend **strongly heavily** on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternate storage methods should be used only after consultation with the receiving laboratory.

Before transfer to the laboratory, pieces of the organs to be examined should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing at least 4 ml transport medium, i.e. cell culture medium with 10% fetal calf serum (FCS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g) and taken in duplicate if retesting may be required.

Tubes containing fish tissues in transport medium for cell cultivation should be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples should be avoided. The temperature of a sample during transit must never exceed 10°C and ice must still be present in the transport box at receipt or one or more freeze blocks must still be partly or completely frozen.

Annex 14 (contd)

Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory. All packaging and labelling must be performed in accordance with ~~present~~ current national and international transport regulations, as appropriate.

The virological examination for isolation in cell culture should be started as soon as possible and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium and that the temperature requirements during transportation can be fulfilled.

3.5.2. Preservation of samples for molecular detection

Samples can be taken from the fish in accordance with the procedure described in Section 3.5.1., using a sterile instrument, and transferred to a sterile plastic tube containing transport medium.

Alternatively, samples may be placed in at least five volumes of RNA stabilisation reagents according to the recommendation from the manufacturers. Samples in RNA stabilising reagents can be shipped on ice or at room temperature if transport time does not exceed 24 hours.

Whole fish may also be sent to the laboratory (see Section 3.5.1.).

Samples may also be frozen at -80°C and kept frozen until assayed (Siah et al., 2014).

3.5.3. Samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Tissue samples for histopathology should be fixed in 10% neutral buffered formalin immediately after collection. The recommended ratio of fixative to tissue is 10:1. To avoid excessive cross-linking, tissue should be transferred to ethanol after 24 hours if methods other than histopathology are used e.g. *in-situ* hybridisation.

3.5.4. Fixed samples for electron microscopy

~~Sampling for electron microscopy should be done according to standard procedures (for an example, see Chapter 2.2.9 Infection with yellow head virus genotype 1). Sampling for electron microscopy is not relevant for diagnostic purposes. Samples for electron microscopy are not routinely required and are collected only when it is considered beneficial to facilitate further diagnostic investigation. A 2 mm cubed section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.~~

3.5.5. Samples for other tests

If samples are processed for ELISA or other immunochemical assays, the procedures described in Section 3.5.1. for pathogen isolation should be followed.

3.6. Pooling of samples

The effect of pooling on diagnostic sensitivity has not been evaluated, therefore, larger fish should be processed and tested individually. However, samples, especially fry or specimens up to 0.5 g, can be pooled to obtain enough material for virus isolation or molecular detection.

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

- +++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;
- ++ = Suitable method(s) but may need further validation;
- + = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;
- Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities, and repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of **apparently** healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts												
Immunohistopathology ³									++	2-++	2-++	2
Histopathology ³						++	++	1				
Cell culture	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Real-time PCR	+++	+++	+++	3	+++	+++	+++	3	+++	+++	+++	3
Conventional RT-PCR	++	++	++	3	+++	++	+++	3	+++	++	+++	3
Amplicon sequencing ⁴									+++	+++	+++	3
<i>In-situ</i> hybridisation												
Bioassay												
LAMP												
Ab-ELISA		+	++	2								
Ag-ELISA									+ ⁵	++ ⁵	++ ⁵	1
IFAT					++	++	++	2	++ ⁵	++ ⁵	++ ⁵	2
Serum neutralisation for Ab detection		+	++	2								

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); RT-PCR = reverse-transcription polymerase chain reaction; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively; IFAT = indirect fluorescent antibody test;

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Early and juvenile life stages have been defined in Section 2.2.4. ³Histopathology and cytopathology can be validated if the results from different operators has been statistically compared. ⁴Sequencing of the PCR product. ⁵only for identification of cultured pathogen. Shading indicates the test is inappropriate or should not be used for this purpose.

Annex 14 (contd)**4.1. Wet mounts**

Not relevant.

4.2. Histopathology and cytopathology

The kidney, liver and spleen show extensive focal necrosis and degeneration – cytoplasmic vacuoles, pyknosis, karyolysis, and lymphocytic invasion. While the skeletal muscle does not appear to be a site of infection, erythrocytes can accumulate in the skeletal muscle bundles and fibres without causing damage to the muscle *per se* (Evensen *et al.*, 1994).

4.3. Cell or artificial media culture for isolation

The recommended cell lines for VHSV detection are bluegill fry (BF-2), epithelioma papulosum cyprini (EPC) or fathead minnow (FHM). Susceptibility of a cell line to VHSV infection will depend on a range of parameters, including cell-line lineage or viral strain differences. Generally, VHSV isolates belonging to either genotypes I, II, or III culture best on BF-2 (Lorenzen *et al.*, 1999), while genotype IV isolates culture best on the EPC cell line (US Department of the Interior, 2007).

4.3.1. Cell lines

Cell lines should be monitored regularly (e.g. every 6 months) to ensure that susceptibility to targeted pathogens has not changed.

Cells are grown at 20–24°C in a suitable medium, e.g. Eagle's minimal essential medium (MEM) (or modifications thereof) with a supplement of 10% fetal bovine serum (FBS) and antibiotics in standard concentrations. When the cells are cultivated in closed vials, it is recommended to buffer the medium with bicarbonate. The medium used for cultivation of cells in open units may be buffered with Tris/HCl (23 mM) and Na-bicarbonate (6 mM), or with HEPES-buffered medium (HEPES=N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid). The pH must be maintained at 7.6 ± 0.2 . Cell cultures to be used for inoculation with tissue material should be young (4–48 hours old) and actively growing (not confluent) at inoculation. Cell susceptibility can be enhanced by reducing the amount of FBS to 2%. Pre-treatment of cells with 7% (w/v) PEG-20,000 solution (10–15 $\mu\text{l}/\text{cm}^2$) 15–30 minutes prior to sample inoculation has also been shown to increase detection of VHSV in culture (Batts *et al.*, 1991).

4.3.2. Sample preparation and inoculation

- i) **Note:** Tissue and fluid samples should be kept cool throughout sample preparation procedures. Homogenise tissue samples using mortar and pestle, ~~stomacher, polytron or equivalent or a tissue homogeniser~~. A small volume of media (MEM-4 or HBSS [Hank's balanced salt solution] + antibiotics) may be needed to achieve complete homogenisation.
- ii) Adjust the volume of media to a final ratio of 10:1 (media:tissue) and mix thoroughly. For fluid samples adjust the volume of media to a final ratio of 1:1.
- iii) Centrifuge the homogenate or fluid samples at 2000–4000 **g** for 15 minutes at 2–5°C.
- iv) Remove the supernatant and pass through a 0.45 μm membrane filter (if available) or treat for either 4 hours at 15°C or overnight at 4°C with antibiotics, e.g. gentamicin 1 mg ml^{-1} .

If the sample cannot be inoculated within 48 hours after collection, the supernatant may be stored at –80°C provided virological examination is carried out within 14 days.

- v) If samples originate from an area where infectious pancreatic necrosis virus (IPNV) is present, supernatants may be treated with IPNV antiserum. Mix the supernatant with equal parts of a suitably diluted pool of antisera to the indigenous serotypes of IPNV and incubate for a minimum of one hour at 15°C or up to 18 hours at 4°C. The titre of the antiserum must be at least 1/2000 in a 50% plaque neutralisation test.

Annex 14 (contd)

Treatment of all inocula with antiserum to IPNV (a virus that in some parts of Europe occurs in 50% of fish samples) aims at preventing cytopathic effect (CPE) caused by IPNV from developing in inoculated cell cultures. This will reduce the duration of the virological examination as well as the number of cases in which occurrence of CPE would have to be considered potentially indicative of VHSV. When samples come from production units that are considered free from infection with IPNV, treatment of inocula with antiserum to IPNV may be omitted.

- vi) Samples are inoculated into cell cultures in at least two dilutions, i.e. the primary dilution and a 1:10 dilution thereof, resulting in final dilutions of tissue material in cell culture medium of 1:100 and 1:1000, respectively. The ratio between inoculum size and volume of cell culture medium should be about 1:10. For each dilution and each cell line, a minimum of about 2 cm² cell area, corresponding to one well in a 24-well cell culture tray, has to be used. Use of cell culture trays is recommended, but other units of similar or with larger growth area are also acceptable.
- vii) Inoculated cell cultures are incubated at 15°C for 7–10 days. Using a microscope with 40–150× magnification, cultures should be inspected for toxicity the day after inoculation, particularly if supernatant was not filtered in step iv. The use of a phase-contrast microscope is recommended.
- viii) Monitor the cells regularly (2–3 times a week) for the presence of CPE.

If CPE is observed, virus identification is required using tests recommended in Section 6. If no CPE is observed after the primary incubation period, subcultivation is performed.

Subcultivation

- i) Remove cell culture supernatant from the primary culture and inoculate a newly (<48 hours) seeded cell culture plate.
- ii) Incubate inoculated plates at 15°C and monitor for 7–10 days as described above.

If CPE is observed, virus identification is required using tests recommended in Section 6. If no CPE is observed after the primary incubation period or subcultivation, the sample is negative.

4.4. Nucleic acid amplification

Use of molecular tests (RT-PCR and real-time RT-PCR) is common because of their rapidity, sensitivity and specificity. Real-time RT-PCR tests are generally more sensitive than conventional RT-PCR tests. These tests for virus detection and identification during the acute stage of disease has been justified for a number of years. At In the acute stage of infection, the sensitivity of some RT-PCR (Kim *et al.*, 2018) and real-time RT-PCR tests (Garver *et al.*, 2011; Jonstrup *et al.*, 2013) is comparable to detection by cell culture and subsequent identification. The molecular methods described in this chapter are all targeting the Nucleoprotein gene, as it is the highest transcribed gene in the VHSV genome (Chico *et al.*, 2006).

Recently, a novel one-step RT-PCR test was developed and validated (Kim *et al.*, 2018) to be used instead of the previously recommended conventional RT-PCR for detecting VHSV. This novel assay has a higher sensitivity detecting all VHSV genotypes, and outperforms the old method, particularly in detecting genotype IV.

For detecting all genotypes of VHSV with real-time RT-PCR, the one-step methods of Jonstrup *et al.* (2013) and Garver *et al.* (2011) have been validated to stage 3 validated, showing a sensitivity similar to detection by cell culture. These methods, have having high analytical and diagnostic sensitivity and specificity, and has been shown to be highly are robust across laboratories (Garver *et al.*, 2011; Jonstrup *et al.*, 2013; Warg *et al.*, 2014a; 2014b).

The following controls should be run with each assay: negative extraction control; positive control; no template control; internal PCR control.

Annex 14 (contd)**4.4.1. Real-time RT-PCR**

Total RNA can be purified from: aliquots of cell culture medium from infected monolayer cells; or tissue/organs homogenised in MEM specified in Section 4.3.1, tissue samples in RNA stabilising reagent, fresh or frozen tissue samples, ovarian fluid.

In the case of culture medium from infected monolayer cells, or in tissue homogenised in MEM, aliquots should be centrifuged at 1000 *g* for 5 minutes to remove cell debris.

One-step (Jonstrup *et al.*, 2013) and two-step (Garver *et al.*, 2011) real-time RT-PCR assays targeting the nucleoprotein gene of VHSV have been stage 3 validated and are described herein.

Positive and negative controls should be included with each stage of the assay: extraction, reverse-transcription (two-step assay only) and real-time PCR. An internal (endogenous) PCR control can be included however given the large number of fish species susceptible to infection with VHSV, the selection of an internal control is not trivial. If an endogenous control is to be used, primers and probes have to be designed, optimised and validated for each fish species to be tested.

Total RNA from infected cells and/or tissues is extracted using a phase-separation method (e.g. phenol-chloroform or Trizol) or by use of a commercially available RNA isolation kit used according to the manufacturer's instructions.

One-step real-time RT-PCR

In one-step RT-PCR gene-specific primers are used both to generate a cDNA transcript and for real-time PCR. Both reactions occur in the same tube, which minimises the probability of contamination. The one-step real-time RT-PCR amplification can be performed using forward primer 5'-AAA-CTC-GCA-GGA-TGT-GTG-CGT-CC-3', reverse primer: 5'-TCT-GCG-ATC-TCA-GTC-AGG-ATG-AA-3', and FAM-labelled probe: 6'-FAM-TAG-AGG-GCC-TTG-GTG-ATC-TTC-TG-BHQ1. Primers are used at a final concentration of 900 nM and the final probe concentration is 250 nM. 5 µl of extracted RNA (50 ng–2 µg) is added to each 25 µl PCR reaction. The assay was validated using Quantitect Probe RT-PCR kit (Qiagen, Germany) following the manufacturer's instructions and is recommended as other one-step kits have demonstrated reduced sensitivity (Jonstrup *et al.*, 2013). Thermal cycling conditions are 50°C for 30 minutes, 95°C for 15 minutes, 40 cycles of 94°C for 15 seconds, 60°C for 40 seconds, 72°C for 20 seconds.

Two-step real-time RT-PCR

i) Step 1: Reverse-transcription

Extracted RNA is reverse transcribed non-discriminately into cDNA using random primers. The cDNA synthesis reactions and cycling conditions are best performed using manufacturer's instructions for commercially available kits which have been extensively tested with a variety of RNA templates, including GC- and AU-rich targets and RNase expressed at low levels.

ii) Step 2: Real-time PCR

The TaqMan real-time PCR assay uses forward primer 5'-ATG-AGG-CAG-GTG-TCG-GAG-G-3', reverse primer 5'-TGT-AGT-AGG-ACT-CTC-CCA-GCA-TCC and FAM-labelled probe 5'-6FAM-TAC-GCC-ATC-ATG-ATG-AGT-MGBNFQ-3'. Primers are used at a final concentration of 600 nM, and the final concentration of the probe is 200 nM. 2.5 µl of cDNA product is added to each 25 µl PCR reaction. Thermal cycling conditions are 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

A sample is negative if no Ct (threshold cycle) is recorded, while samples with a Ct are considered positive for VHSV. Cut-off value depends on the set-up in each laboratory but is usually set at Ct ≥ 40).

4.4.2. Conventional PCR

RNA isolation is done as in Section 4.4.1. Positive and negative controls should be run with each stage of the assays: extraction, RT-PCR and second round PCR. Due to the sensitive nature of PCR-based assays it is highly recommended that master mix, template addition and PCR amplification occur in designated hoods or spatially separated areas.

A one-step RT-PCR should be performed as described by Kim *et al.* (2018) with 3F2R primer set: forward primers (3F, 5'-(GGG-ACA-GGA-ATG-ACC-ATG-AT-3') and reverse primer (2R, (5'-TCT-GTC-ACC-TTG-ATC-CCC-TCC-AG-3') targeting a 319 nt region in the nucleoprotein gene (positions 658–977).

The RT-PCR can be performed using, e.g. Qiagen OneStep RT-PCR System (Qiagen, Germany) or similar kit, according to the manufacturer's instructions. Briefly, the reaction mixture is adjusted to a final volume of 25 µl including 5 µl of extracted viral RNA, 5 µl 5 × One Step RT-PCR Buffer containing 12.5 mM MgCl₂ (final concentration 2.5 mM), 10 pM of each primer, and 1 µl of enzyme mix.

The following cycles are recommended: 50°C for 30 minutes, 95°C for 15 minutes, 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 60 seconds. Subsequently, the reaction is held at 68°C for 7 minutes.

4.4.3. Other nucleic acid amplification methods

To date, no other nucleic acid amplification method capable of universal VHSV detection has been sufficiently validated.

4.5. Amplicon sequencing

Nucleotide sequencing of the glycoprotein gene is commonly used for identification of genetic strains and for epidemiological study and is recommended as one of the final steps for confirmatory diagnosis. There are several conventional PCR assays available that amplify the central (669 nt) or full (1524 nt) glycoprotein gene coding sequence, but there are limited validation data. The glycoprotein gene can be amplified by conventional PCR using the primer sets and concentrations listed in Table 4.2. The reverse transcription and subsequent PCR amplification can be done using a kit designed for that purpose according to manufacturing instructions.

Table 4.2. Primer sets for the conventional PCR

Primer	Sequence (5'–3')	Product size (bp)	Final primer concentration	Reference
GB+	GTC-GAA-GAA-GAG-ATA-GGC	1757	0.6 µM	Einer-Jensen <i>et al.</i> , 2004 Gudmundsdottir <i>et al.</i> , 2019
GB-	GTT-GGG-TCG-CCA-TGT-TTC-T		0.6 µM	
G330+	ACT-ACC-TAC-ACA-GAG-TGA-C	914	0.2 µM	Garver <i>et al.</i> , 2013
G1243-	CAA-TTT-GTC-CCC-GAA-TAT-CAT		0.2 µM	
G422+	TCC-CGT-CAA-GAG-GCC-AC	669	0.2 µM	
G1179-	TTC-CAG-GTG-TTG-TTT-ACC-G		0.2 µM	

4.6. In-situ hybridisation

Not relevant in relation to primary diagnosis and surveillance of infection with VHSV.

4.7. Immunohistochemistry

Immunohistochemistry reveals VHSV-positive endothelial cells, primarily in the vascular system (Evensen *et al.*, 1994). Specific polyclonal and monoclonal antibodies for immunohistochemistry are commercially available.

4.8. Bioassay

Not relevant in relation to primary diagnostics and surveillance of infection with VHSV.

4.9. Antibody- or antigen-based detection methods

Antibody- and antigen-based detection methods should not be used as a method of screening healthy populations.

Annex 14 (contd)**4.9.1. Antigen enzyme-linked immunosorbent assay (ELISA)**

- i) Coat the wells of microplates designed for enzyme-linked immunosorbent assays (ELISAs) with appropriate dilutions of protein-A purified immunoglobulins (Ig) from rabbit anti sera against VHSV in carbonate buffer, pH 9.6 (50 $\mu\text{l well}^{-1}$).
- ii) Incubate overnight at 4°C.
- iii) Rinse in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST).
- iv) Add 1% Triton X-100 to the virus suspension to be identified.
- v) Dispense 50 $\mu\text{l well}^{-1}$ of two- or four-step dilutions (in PBST containing 1% bovine serum albumin) of the virus to be identified and of VHSV control virus, as well as a negative control (e.g. infectious haematopoietic necrosis virus [IHNV]), and allow to react with the coated antibody to VHSV for 1 hour at 37°C.
- vi) Rinse in PBST.
- vii) Add to the wells monoclonal antibodies to VHSV N protein (IP5B11) 50 $\mu\text{l well}^{-1}$.
- viii) Incubate for 1 hour at 37°C.
- ix) Rinse in PBST.
- x) Add to the wells (50 $\mu\text{l well}^{-1}$) horseradish peroxidase (HRP)-conjugated monoclonal anti-mouse antibodies.
- xi) Incubate for 1 hour at 37°C.
- xii) Rinse in PBST.
- xiii) Visualise the reaction using TMB (3,3',5,5'-tetramethylbenzidine) and measure the absorbance at a wavelength of 450 nm.

The above ELISA version is given as an example. Other ELISA versions of demonstrated performance may be used instead.

For positive controls, use cell culture supernatant from cultures inoculated with known VHSV isolate.

For negative controls, use cell culture supernatant from same cell line inoculated with heterologous virus (e.g. IHNV) or from non-infected culture.

4.9.2. Indirect fluorescent antibody test (IFAT)

- i) Prepare monolayers of cells in 2 cm^2 wells of cell culture plastic plates or on cover-slips to reach around 80% confluence, which is usually achieved within 24 hours of incubation at 22°C (seed six cell monolayers per virus isolate to be identified, plus two for positive and two for negative controls). The FCS content of the cell culture medium can be reduced to 2–4%. If numerous virus isolates have to be identified, the use of Terasaki plates is strongly recommended.
- ii) When the cell monolayers are ready for infection, i.e. on the same day or on the day after seeding, inoculate the virus suspensions to be identified by making tenfold dilution steps directly in the cell culture wells or flasks.
- iii) Dilute the control virus suspension of VHSV in a similar way, in order to obtain a virus titre of about 5000–10,000 plaque-forming units (PFU) ml^{-1} in the cell culture medium.
- iv) Incubate at 15°C for 24 hours.

Annex 14 (contd)

- v) Remove the cell culture medium, rinse once with 0.01 M PBS, pH 7.2, then three times briefly with a cold mixture of acetone 30% and ethanol 70% (v/v) (stored at -20°C).
- vi) Let the fixative act for 15 minutes. A volume of 0.5 ml is adequate for 2 cm^2 of cell monolayer.
- vii) Allow the cell monolayers to air-dry for at least 30 minutes and process immediately or freeze at -20°C .
- viii) Prepare a solution of purified VHSV antibody or serum in 0.01 M PBST, pH 7.2, at the appropriate dilution (which has been established previously or is given by the reagent supplier).
- ix) Rehydrate the dried cell monolayers by using four rinsing steps with the PBST solution and remove this buffer completely after the last rinse.
- x) Treat the cell monolayers with the antibody solution for 1 hour at 37°C in a humid chamber and do not allow evaporation to occur, e.g. by adding a piece of wet cotton in the humid chamber. The volume of solution to be used is 0.25 ml per 2 cm^2 well.
- xi) Rinse four times with PBST as above.
- xii) Treat the cell monolayers for 1 hour at 37°C with a solution of fluorescein isothiocyanate (FITC)- or tetramethylrhodamine-5-(and-6-) isothiocyanate (TRITC)-conjugated antibody to the immunoglobulin used as the primary antibody and prepared according to the instructions of the supplier. These conjugated antibodies are most often rabbit or goat antibodies.
- xiii) Rinse four times with PBST.
- xiv) Examine the treated cell monolayers on plastic plates immediately, or mount the cover-slips using, for example glycerol saline, pH 8.5 prior to microscopic observation.
- xv) Examine under incident UV light using a microscope with $\times 10$ eye pieces and $\times 20$ – 40 objective lens having numerical aperture >0.65 and >1.3 respectively. Positive and negative controls must yield the expected results prior to any other observation.

Other IFAT or immunocytochemical (alkaline phosphatase or peroxidase) techniques of demonstrated performance may be used instead.

Always include positive control such as wells or coverslip with cells infected with a known VHSV isolate.

4.10. Other serological methods

4.10.1. Neutralisation test

- i) Collect the culture medium of the cell monolayers exhibiting CPE and centrifuge it at 2000 g for 15 minutes at 4°C , or filter through a $0.45\text{ }\mu\text{m}$ (or 450 nm) pore membrane to remove cell debris.
- ii) Dilute virus-containing medium from 10^{-2} to 10^{-4} .
- iii) Mix aliquots (for example $200\text{ }\mu\text{l}$) of each dilution with equal volumes of a VHSV antibody solution and, likewise, treat aliquots of each virus dilution with cell culture medium. The neutralising antibody [NAb] solution must have a 50% plaque reduction titre of at least 2000.
- iv) In parallel, another neutralisation test must be performed against a homologous virus strain (positive neutralisation test).
- v) If required, a similar neutralisation test may be performed using antibodies to IPNV.
- vi) Incubate all the mixtures at 15°C for 1 hour.

UNOFFICIAL VERSION

Annex 14 (contd)

- vii) Transfer aliquots of each of the above mixtures on to 24–48 hour-old monolayers, overlaid with cell culture medium containing 10% FCS (inoculate two wells per dilution), and incubate at 15°C; 24- or 12-well cell culture plates are suitable for this purpose, using a 50 µl inoculum.
- viii) Check the cell cultures for the onset of CPE and read the result as soon as it occurs in non-neutralised controls (cell monolayers being protected in positive neutralisation controls). Results are recorded either after a simple microscopic examination (phase contrast preferable) or after discarding the cell culture medium and staining cell monolayers with a solution of 1% crystal violet in 20% ethanol.
- ix) The tested virus is identified as VHSV when CPE is prevented or noticeably delayed in the cell cultures that received the virus suspension treated with the VHSV-specific antibody, whereas CPE is evident in all other cell cultures.
- x) In the absence of any neutralisation by NAb to VHSV, it is mandatory to conduct an RT-PCR, an ELISA or IFAT, using the suspect sample. Some cases of antigenic drift of surface antigen have been observed, resulting in occasional failure of the neutralisation test using NAb to VHSV.

Other neutralisation tests of demonstrated performance may be used instead.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Virus isolation, real-time RT-PCR and conventional PCR are the recommended tests for surveillance to demonstrate freedom of disease in apparently healthy population.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status⁴

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link to an infected population. Geographical proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with VHSV shall be suspected if at least one of the following criteria is met:

- i) VHSV-typical CPE in cell cultures before confirmation;
- ii) A positive result from a real-time PCR assay;
- iii) A positive result from a conventional PCR assay;
- iv) Detection of antibodies (by Ab-ELISA or serum neutralisation in adults only).

⁴ For example transboundary *commodities*.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with VHSV is considered to be confirmed if, in addition to the criteria in Section 6.1.1., one or more of the following criteria is met:

- ~~i) VHSV isolation in cell culture followed by virus identification by conventional RT-PCR, and by sequencing of the amplicon;~~
- ~~ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by conventional RT-PCR and sequencing of the amplicon;~~
- ~~iii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;~~
- ~~iv-iii) Detection of VHSV in tissue preparations by real-time RT-PCR, and by a conventional RT-PCR (targeting a non-overlapping region of the genome) and sequencing of the amplicon~~

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.2 Clinically affected animals

No clinical signs are pathognomonic for infection with VHSV however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with VHSV shall be suspected if at least one of the following criteria is met:

- i) Gross pathology or clinical signs associated with infection with VHSV as described in this chapter, with or without elevated mortality;
- ii) Histopathological changes consistent with infection with VHSV as described in this chapter;
- iii) A positive result from real-time PCR, ~~conventional PCR, or IFAT;~~
- iv) A positive result from a conventional PCR;
- v) A positive result by IFAT
- vi) VHSV-typical CPE-Cytopathic effect in cell culture.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with VHSV shall be confirmed if, in addition to the criteria in Section 6.2.1., positive results has been obtained on at least one animal from two tests used in the following combination-one or more of the following criteria is met:

- i) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by conventional RT-PCR and sequencing of the amplicon;
- ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;
- iii) Detection of VHSV in tissue preparations by real-time RT-PCR, and by a conventional RT-PCR (targeting a non-overlapping region of the genome) and sequencing of the amplicon.
- ~~i) VHSV isolation in cell culture, followed by virus identification by conventional RT-PCR, and sequencing of the amplicon;~~
- ~~ii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by conventional RT-PCR and sequencing of the amplicon;~~

Annex 14 (contd)

- iii) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and detection of VHSV in tissue preparations by real-time RT-PCR;
- iv) VHSV isolation in cell culture, followed by virus identification by real-time RT-PCR, Ag-ELISA, or IFAT and a positive result from immunohistopathology);
- v) Detection of VHSV in tissue preparations by real-time RT-PCR and by conventional RT-PCR, followed by sequencing of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with VHSV are provided in Table 6.3. This information can be used for the design of surveys for infection with VHSV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

Table 6.1. Diagnostic performance of tests recommended for surveillance or diagnosis

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (n)	DSp (n)	Reference test	Citation
Cell culture	Surveillance	Experimentally infected fish	Kidney, heart and spleen	Rainbow trout	86 (84)	–	Real-time RT-PCR	Jonstrup <i>et al.</i> , 2013
Cell culture	Clinical diagnosis	Experimentally infected fish	Kidney	Atlantic salmon	100 (100)	94.4 (100)	Pseudo-gold standard*	Garver <i>et al.</i> , 2011
Real-time RT-PCR	Surveillance	Experimentally infected fish	Kidney	Atlantic salmon	93 (30)	100 (70)	Cell culture	Garver <i>et al.</i> , 2011
Real-time RT-PCR	Surveillance	Experimentally infected fish	Kidney, heart and spleen	Rainbow trout	90 (84)	100 (43)	Cell culture	Jonstrup <i>et al.</i> , 2013

* a compilation of 8 test results to evaluate both the RT-qPCR and virus isolation assay (Garver *et al.*, 2011);

DSe = diagnostic sensitivity, DSp = diagnostic specificity; *n* = number of samples used in the study.

7. References

AHNE W. (1982). Vergleichende Untersuchung über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV) (Comparative studies on the stability of four fish-pathogenic viruses [VHSV, PFR, SVCV, IPNV]). *Zentralbl. Veterinarmed. [B]*, **29**, 457–476. (In German).

ALTUNTAS C. & OGUT H. (2010). Monthly occurrence and prevalence of viral haemorrhagic septicaemia virus (VHSV) in whiting *Merlangius merlangus*. *Dis. Aquat. Org.*, **88**, 107–113.

ARKUSH K.D., MENDONCA H.L., MCBRIDE A.M., YUN S., MCDOWELL T.S. & HEDRICK R.P. (2006). Effects of temperature on infectivity and of commercial freezing on survival of the North American strain of viral hemorrhagic septicemia virus (VHSV). *Dis. Aquat. Org.*, **69**, 145–151.

AUINGER B.M., PFANDL K. & BOENIGK J. (2008). Improved methodology for identification of protists and microalgae from plankton samples preserved in Lugol's iodine solution: combining microscopic analysis with single-cell PCR. *Appl. Environ. Microbiol.*, **74**, 2505–2510.

BATTS W.N., TRAXLER G.S. & WINTON J.R. (1991). Factors affecting the efficiency of plating for selected fish rhabdoviruses. *In: Proceeding of the 2nd International Symposium on Viruses of Lower Vertebrates*, Fryer J.L., ed. July 29–31, Oregon University, Corvallis, OR, 17–24.

BOVO G., HÅSTEIN T., HILL B., LAPATRA S., MICHEL C., OLESEN N.J., SHCHELKUNOV I., STORSET A., WOLLFROM T. & MIDTLYNG P.J. (2005a). QLK2-CT-2002-01546: Fish Egg Trade Work package 1 report: Hazard identification for vertical transfer of fish disease agents, 1–35. VESO, P.O. Box 8109, Dep., N-0032 Oslo, Norway.

BOVO G., HILL B., HUSBY A., HÅSTEIN T., MICHEL C., OLESEN N.J., STORSET A. & MIDTLYNG P. (2005b). Fish Egg Trade Work package 3 report: Pathogen survival outside the host, and susceptibility to disinfection, 1–53. VESO, P.O. Box 8109 Dep., N-0032 Oslo, Norway. Available at: http://www.eurl-fish.eu/-/media/Sites/EURL-FISH/english/activities/scientific%20reports/fisheggtrade%20wp_3.ashx?la=da

CASTRIC J. & DE KINKELIN P. (1984). Experimental study of the susceptibility of two marine fish species, sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*), to viral haemorrhagic septicaemia. *Aquaculture*, **41**, 203–212.

CHICO V., GOMEZ N., ESTEPA A. & PEREZ L. (2006). Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time RT-PCR. *J. Virol. Methods*, **132**, 154–159.

CORNWELL E.R., BELLMUND C.A., GROOCOCK G.H., WONG P.T., HAMBURY K.L., GETCHELL R.G. & BOWSER P.R. (2013). Fin and gill biopsies are effective nonlethal samples for detection of viral hemorrhagic septicemia virus genotype IVb. *J. Vet. Diagn. Invest.*, **25**, 203–209.

DALE O.B., ORPETVEIT I., LYNGSTAD T.M., KAHNS S., SKALL H.F., OLESEN N.J. & DANNEVIG B.H. (2009). Outbreak of viral haemorrhagic septicaemia (VHS) in seawater-farmed rainbow trout in Norway caused by VHS virus Genotype III. *Dis. Aquat. Org.*, **85**, 93–103.

DE KINKELIN P. & LE BERRE M. (1977). Isolation of a pathogenic rhabdovirus of brown trout (*Salmo trutta* L., 1766). *C.R. Hebd. Séances Acad. Sci.*, **284**, 101–104.

DORSON M., QUILLET E., HOLLEBECQ M.G., TORHY C. & CHEVASSUS B. (1995). Selection of rainbow trout resistant to viral haemorrhagic septicaemia virus and transmission of resistance by gynogenesis. *Vet. Res.*, **26**, 361–368.

EINER-JENSEN K., AHRENS P., FORSBERG R. & LORENZEN N. (2004). Evolution of the fish rhabdovirus viral haemorrhagic septicaemia virus. *J. Gen. Virol.*, **85**, 1167–1179.

ELSAIED E., FAISAL M., THOMAS M., WHELAN G., BATTS W. & WINTON J. (2006). Isolation of viral haemorrhagic septicaemia virus from muskellunge, *Esox masquinongy* (Mitchill), in Lake St Clair, Michigan, USA reveals a new sublineage of the North American genotype. *J. Fish Dis.*, **29**, 611–619.

EVENSEN Ø., MEIER W., WAHLI T., OLESEN N.J., JØRGENSEN P.E.V. & HÅSTEIN T. (1994). Comparison of immunohistochemistry and virus cultivation for detection of viral haemorrhagic septicaemia virus in experimentally infected rainbow trout *Oncorhynchus mykiss*. *Dis. Aquat. Org.*, **20**, 101–109.

FAISAL M. & SCHULZ C.A. (2009). Detection of Viral hemorrhagic septicemia virus (VHSV) from the leech *Myzobdella lugubris* Leidy, 1851. *Parasit. Vectors*, **2**, 45.

FAISAL M. & WINTERS A.D. (2011). Detection of viral hemorrhagic septicemia virus (VHSV) from *Diporeia* spp. (Pontoporeiidae, Amphipoda) in the Laurentian Great Lakes, USA. *Parasit. Vectors*, **4**, 2.

GADD T., JAKAVA-VILJANEN M., EINER-JENSEN K., ARIEL E., KOSKI P. & SIHVONEN L. (2010). Viral haemorrhagic septicaemia virus (VHSV) genotype II isolated from European river lamprey *Lampetra fluviatilis* in Finland during surveillance from 1999 to 2008. *Dis. Aquat. Org.*, **88**, 189–198.

GADD T., JAKAVA-VILJANEN M., TAPIOVAARA H., KOSKI P. & SIHVONEN L. (2011). Epidemiological aspects of viral haemorrhagic septicaemia virus genotype II isolated from Baltic herring, *Clupea harengus membras* L. *J. Fish Dis.*, **34**, 517–529.

Annex 14 (contd)

GAGNE N., MACKINNON A.M., BOSTON L., SOUTER B., COOK-VERSLOOT M., GRIFFITHS S., & OLIVIER G. (2007). Isolation of viral haemorrhagic septicaemia virus from mummichog, stickleback, striped bass and brown trout in eastern Canada. *J. Fish Dis.*, **30**, 213–223.

GARVER K.A., HAWLEY L.M., McCLURE C.A., SCHROEDER T., ALDOUS S., DOIG F., SNOW M., EDES S., BAYNES C. & RICHARD J. (2011). Development and validation of a reverse transcription quantitative PCR for universal detection of viral hemorrhagic septicemia virus. *Dis. Aquat. Org.*, **95**, 97–112.

GARVER K.A., TRAXLER G.S., HAWLEY L.M., RICHARD J., ROSS J.P. & LOVY J. (2013). Molecular epidemiology of viral haemorrhagic septicaemia virus (VHSV) in British Columbia, Canada, reveals transmission from wild to farmed fish. *Dis. Aquat. Org.*, **104**, 93–104. doi: 10.3354/dao02588.

GOODWIN A.E. & MERRY G.E. (2011). Mortality and carrier status of bluegills exposed to viral hemorrhagic septicemia virus genotype IVb at different temperatures. *J. Aquat. Anim. Health*, **23**, 85–91.

GROSS L., RICHARD J., HERSHBERGER P. & GARVER K. (2019). Low susceptibility of sockeye salmon *Oncorhynchus nerka* to viral hemorrhagic septicemia virus genotype IVa. *Dis. Aquat. Org.*, **135**, 201–209. doi: 10.3354/dao03398

GUDMUNDSDOTTIR S., VENDRAMIN N., CUENCA A., SIGURÐARDOTTIR H., KRISTMUNDSSON A., IBURG T.M. & OLESEN N.J. (2019). Outbreak of viral haemorrhagic septicaemia (VHS) in lumpfish (*Cyclopterus lumpus*) in Iceland caused by VHS virus Genotype IV. *J. Fish Dis.*, **42**, 47–62. <https://doi.org/10.1111/jfd.12910>

HAWLEY L.M. & GARVER K.A. (2008). Stability of viral hemorrhagic septicemia virus (VHSV) in freshwater and seawater at various temperatures. *Dis. Aquat. Org.*, **82**, 171–178.

HENRYON M., BERG P., OLESEN N.J., KJÆR T.E., SLIERENDRECHT W.J., JOKUMSEN A. & LUND I. (2005). Selective breeding provides an approach to increase resistance of rainbow trout to the diseases, enteric redmouth disease, rainbow trout fry syndrome, and viral haemorrhagic septicaemia. *Aquaculture*, **250**, 621–636.

HENRYON M., JOKUMSEN A., BERG P., LUND I., PEDERSEN P.B., OLESEN N.J. & SLIERENDRECHT W.J. (2002a). Erratum to “Genetic variation for growth rate, feed conversion efficiency, and disease resistance exists within a farmed population of rainbow trout” (*Aquaculture*, 2002, **209**, 59–76). *Aquaculture*, **216**, 389–390.

HENRYON M., JOKUMSEN A., BERG P., LUND I., PEDERSEN P.B., OLESEN N.J. & SLIERENDRECHT W.J. (2002b). Genetic variation for growth rate, feed conversion efficiency, and disease resistance exists within a farmed population of rainbow trout. *Aquaculture*, **209**, 59–76.

HERSHBERGER P., GREGG J., GRADY C., COLLINS R. & WINTON J. (2010). Kinetics of viral shedding provide insights into the epidemiology of viral hemorrhagic septicemia in Pacific herring. *Marine Ecology-Progress Series*, 400:187e93.

ISSHIKI T., NISHIZAWA T., KOBAYASHI T., NAGANO T. & MIYAZAKI T. (2001). An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. *Dis. Aquat. Org.*, **47**, 87–99.

ITO T. & OLESEN N.J. (2017). Viral haemorrhagic septicaemia virus (VHSV) remains viable for several days but at low levels in the water flea *Moina macrocopa*. *Dis. Aquat. Org.*, **127**, 11–18.

JENSEN N.J. & LARSEN J.L. (1979). The Ulcus-syndrome in cod (*Gadus morhua*). I. A pathological and histopathological study. *Nord Vet. Med.*, **31**, 222–228.

JOHANSEN R., BERGH Ø., MODAHL I., DAHLE G., GJERSET B., HOLST J.C., SANDLUND N. (2013). High prevalence of viral haemorrhagic septicaemia virus (VHSV) in Norwegian spring-spawning herring. *Mar. Ecol. Prog. Ser.*, **478**, 223–230.

JONSTRUP S.P., GRAY T., KAHNS S., SKALL H.F., SNOW M. & OLESEN N.J. (2009). FishPathogens.eu/vhsv: a user-friendly viral haemorrhagic septicaemia virus isolate and sequence database. *J. Fish Dis.*, **32**, 925–929. www.fishpathogens.eu

JONSTRUP S.P., KAHNS S., SKALL H.F., BOUTRUP T.S. & OLESEN N.J. (2013). Development and validation of a novel Taqman based real time RT-PCR assay suitable for demonstrating freedom from viral haemorrhagic septicaemia virus. *J. Fish Dis.*, **36**, 9–23.

KAHNS S., SKALL H.F., KAAS R.S., KORSHOLM H., JENSEN B.B., JONSTRUP S.P., DODGE M.J., EINER-JENSEN K., STONE D. & OLESEN, N. J. (2012). European freshwater VHSV genotype Ia isolates divide into two distinct subpopulations. *Dis. Aquat. Org.*, **99**, 23–35.

KALAYCI G., INCOGLU S. & OZKAN B. (2006). First isolation of viral haemorrhagic septicaemia (VHS) virus from turbot (*Scophthalmus maximus*) cultured in the Trabzon coastal area of the Black Sea in Turkey. *Bull. Eur. Ass. Fish Pathol.*, **26**, 157–162.

KIM H.J., CUENCA A. & OLESEN N.J. (2018). Validation of a novel one-step reverse transcription polymerase chain reaction method for detecting viral haemorrhagic septicaemia virus. *Aquaculture*, **492**, 170–183.

KOCAN R.M., HERSHBERGER P.K., ELDER N.E. & WINTON J.R. (2001). Survival of the North American strain of viral hemorrhagic septicemia virus (VHSV) in filtered seawater and seawater containing ovarian fluid, crude oil and serum-enriched culture medium. *Dis. Aquat. Org.*, **44**, 75–78.

LOPEZ-VAZQUEZ C., DOPAZO C.P., OLVEIRA J.G., BARJA I. & BANDÍN J.L. (2006a). Development of a rapid, sensitive and non-lethal diagnostic assay for the detection of viral haemorrhagic septicaemia virus *J. Virol. Methods*, **133**, 167–174.

LOPEZ-VAZQUEZ C., RAYNARD R.S., BAIN N., SNOW M., BANDÍN I. & DOPAZO C.P. (2006b). Genotyping of marine viral haemorrhagic septicaemia virus isolated from the Flemish Cap by nucleotide sequence analysis and restriction fragment length polymorphism patterns. *Dis. Aquat. Org.* **73**, 23–31.

LORENZEN E., CARSTENSEN B. & OLESEN N.J. (1999). Inter-laboratory comparison of cell lines for susceptibility to three viruses: VHSV, IHNV and IPNV. *Dis. Aquat. Org.*, **37**, 81–88.

LORENZEN N. & LAPATRA S.E. (2005). DNA vaccines for aquacultured fish. *Rev. sci. tech. Off. int. Epiz.*, **24**, 201–213.

MEIER W. & JORGENSEN P.E.V. (1980). Isolation of VHS virus from pike fry (*Esox lucius*) with hemorrhagic symptoms. In: *Fish Diseases, Third COPRAQ Session*, Ahne W., ed. Springer-Verlag, Berlin, Germany and New York, USA, 8–17.

MEYERS T.R., SULLIVAN J., EMMENEGGER E., FOLLETT J., SHORT S., BATTS W.N. & WINTON J.R. (1992). Identification of viral haemorrhagic septicaemia virus from Pacific cod *Gadus macrocephalus* in Prince William Sound, Alaska, USA. *Dis. Aquat. Org.*, **12**, 167–175.

MEYERS T.R. & WINTON J.R. (1995). Viral haemorrhagic septicaemia virus in North America. *Ann. Rev. Fish Dis.*, **5**, 3–24.

MUNRO E.S. & GREGORY A. (2010). The risk associated with vertical transmission of viral haemorrhagic septicaemia virus (VHSV) in rainbow trout (*Oncorhynchus mykiss*) eggs. *Bull. Eur. Ass. Fish Pathol.*, **30**, 154–158.

MUNRO E.S., MCINTOSH R.E., WEIR S.J., NOGUERA P.A., SANDILANDS J.M., MATEJUSOVA I., MAYES A.S. & SMITH R. (2015). A mortality event in wrasse species (Labridae) associated with the presence of viral haemorrhagic septicaemia virus *J. Fish Dis.*, **38**, 335–341.

NISHIZAWA T., IIDA H., TAKANO R., ISSHIKI T., NAKAJIMA K. & MUROGA K. (2002). Genetic relatedness among Japanese, American and European isolates of viral haemorrhagic septicaemia virus (VHSV) based on partial G and P genes. *Dis. Aquat. Org.*, **48**, 143–148.

NISHIZAWA T., SAVAS H., ISIDAN H., ÜSTÜNDAG C., IWAMOTO H. & YOSHIMIZU M. (2006). Genotyping and pathogenicity of viral hemorrhagic septicemia virus from free-living turbot (*Psetta maxima*) in a Turkish coastal area of the Black Sea. *Appl. Environ. Microbiol.*, **72**, 2373–2378.

Annex 14 (contd)

NORDBLOM B. (1998) Report on an Outbreak of Viral Haemorrhagic Septicaemia in Sweden. Report for the Standing Veterinary Committee, Swedish Board of Agriculture, Department for Animal Production and Health.

NORDBLOM B. & NORELL A.W. (2000). Report on an Outbreak of VHS (Viral Hemorrhagic Septicaemia) in Farmed Fish in Sweden. Report for the Standing Veterinary Committee, Swedish Board of Agriculture, Department for Animal Production and Health.

OGUT H. & ALTUNTAS C. (2014). Survey of viral haemorrhagic septicaemia virus in wild fishes in the southeastern Black Sea. *Dis. Aquat. Org.*, **109**, 99–106. doi: 10.3354/dao02728.

OIDTMANN B., JOINER C., REESE R.A., STONE D., DODGE M. & DIXON P. (2011). Risks Associated with Commodity Trade: Transmission of Viral Haemorrhagic Septicaemia Virus (VHSV) to Rainbow Trout Fry from VHSV-Carrying Tissue-Homogenates. *Transbound. Emerg. Dis.*, **58**, 224–231.

OLESEN N.J. & JØRGENSEN P.E. (1982). Can and do herons serve as vectors for Egtved virus? *Bull. Eur. Ass. Fish Pathol.*, **2**, 48.

PARRY L. & DIXON P.F. (1997). Stability of nine viral haemorrhagic septicaemia virus (VHSV) isolates in seawater. *Bull. Eur. Ass. Fish Pathol.*, **17**, 31–36.

PEDDIE S., MCLAUCHLAN P.E., ELLIS A.E. & SECOMBES C.J. (2003). Effect of intraperitoneally administered IL-1b-derived peptides on resistance to viral haemorrhagic septicaemia in rainbow trout *Oncorhynchus mykiss*. *Dis. Aquat. Org.*, **56**, 195–200.

PETERS F. & NEUKIRCH M. (1986). Transmission of some fish pathogenic viruses by the heron, *Ardea cinerea*. *J. Fish Dis.*, **9**, 539–544.

PHAM P.H., SOKEECHAND B.S.H., GARVER K.A., JONES G., LUMSDEN J.S. & BOLLS N.C. (2018). Fish viruses stored in RNAlater can remain infectious and even be temporarily protected from inactivation by heat or by tissue homogenates. *J. Virol. Methods*, **253**, 31–37.

PIERCE L.R. & STEPIEN C.A. (2012). Evolution and biogeography of an emerging quasispecies: Diversity patterns of the fish Viral Hemorrhagic Septicemia virus (VHSV). *Mol. Phyl. Evol.*, **63**, 327–341.

RAJA-HALLI M., VEHMAS T.K., RIMAILA-PÄRNÄNEN E., SAINMAA S., SKALL H.F., OLESEN N.J. & TAPIOVAARA H. (2006). Viral haemorrhagic septicaemia (VHS) outbreaks in Finnish rainbow trout farms. *Dis. Aquat. Org.*, **72**, 201–211.

SANDLUND N., GJERSET B., BERGH Ø., MODAHL I., OLESEN N.J. & JOHANSEN R. (2014). Screening for Viral Hemorrhagic Septicemia Virus in Marine Fish along the Norwegian Coastal Line. *PLoS ONE* 9(9): e108529. doi:10.1371/journal.pone.0108529

SCHLOTTFELDT H.J. & AHNE W. (1988). Epizootics in brown trout (*Salmo trutta fario*) caused by VHSV F1. *J. Appl. Ichthyol.*, **4**, 147–148.

SCHLOTTFELDT H.-J., AHNE W., JØRGENSEN P.E.V. & GLENDE W. (1991). Occurrence of viral haemorrhagic septicaemia in turbot (*Scophthalmus maximus*) – a natural outbreak. *Bull. Eur. Assoc. Fish Pathol.*, **11**, 105–107.

SCHONHERZ A.A., HANSEN M.H., JØRGENSEN H.B., BERG P., LORENZEN N. & EINER-JENSEN K. (2012). Oral transmission as a route of infection for viral haemorrhagic septicaemia virus in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.*, **35**, 395–406.

SIAH A., DUESEND H., FRISCH H., NYLUND A., MCKENZIE & SAKSIDA S. (2014). Development of a Multiplex Assay to Measure the Effects of Shipping and Storage Conditions on the Quality of RNA Used in Molecular Assay for Detection of Viral Haemorrhagic Septicemia Virus. *J. Aquat. Anim. Health*, **26**, 173–180.

SKALL H.F., JØRGENSEN C. & OLESEN N.J. (2015). Evaluation of the effect of percolation and NaCl solutions on viral haemorrhagic septicaemia virus (VHSV) under experimental conditions. *Aquaculture*, **448**, 507–511.

SKALL H.F., OLESEN N.J. & MELLERGAARD S. (2005a). Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming – a review. *J. Fish Dis.*, **28**, 509–529.

Annex 14 (contd)

SKALL H.F., OLESEN N.J. & MELLERGAARD S. (2005b). Prevalence of viral haemorrhagic septicaemia virus in Danish marine fishes and its occurrence in new host species. *Dis. Aquat. Org.*, **66**, 145–151.

SKALL H.F., SLIERENDRECHT W.J., KING J.A. & OLESEN N.J. (2004). Experimental infection of rainbow trout (*Oncorhynchus mykiss*) with viral haemorrhagic septicaemia virus isolates from European marine and farmed fishes. *Dis. Aquat. Org.*, **58**, 99–110.

SMAIL D.A. (2000). Isolation and identification of Viral Haemorrhagic Septicaemia (VHS) viruses from cod *Gadus morhua* with ulcus syndrome and from haddock *Melanogrammus aeglefinus* having skin haemorrhages in the North Sea. *Dis. Aquat. Org.*, **41**, 231–235.

SMAIL D.A. & SNOW M. (2011). Viral Haemorrhagic Septicaemia. In: Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections, Second Edition, Woo P.T.K. & Bruno D.W. eds. CABI, Wallingford, UK, 110–142.

SNOW M., BAIN N., BLACK J., TAUPIN V., CUNNINGHAM C.O., KING J.A., SKALL H.F. & RAYNARD R.S. (2004). Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). *Dis. Aquat. Org.*, **61**, 11–21.

STAPIEN C.A., PIERCE L.R., LEAMAN D.W., NINER M.D. & SHEPHERD B.S. (2015). Gene Diversification of an Emerging Pathogen: A Decade of Mutation in a Novel Fish Viral Hemorrhagic Septicemia (VHS) Substrain since Its First Appearance in the Laurentian Great Lakes. *PLoS one*, 1–25.

THOMPSON T.M., BATTS W.N., FAISAL M., BOWSER P., CASEY J.W., PHILLIPS K., GARVER K.A., WINTON J. & KURATH G. (2011). Emergence of Viral hemorrhagic septicemia virus in the North American Great Lakes region is associated with low viral genetic diversity. *Dis. Aquat. Org.*, **96**, 29–43.

THROCKMORTON E., BRENDEN T., PETERS A.K., NEWCOMB T.J., WHELAN G.E. & FAISAL M. (2017). Potential Reservoirs and Risk Factors for VHSV IVb in an Enzootic System: Budd Lake, Michigan. *J. Aquat. Anim. Health*, **29**, 31–42. doi: 10.1080/08997659.2016.1254121

TOPLAK I., HOSTNIK P., RIHTARIČ D., OLESEN N.J., SKALL H.F. & JENČIČ V. (2010). First isolation and genotyping of viruses from recent outbreaks of viral haemorrhagic septicaemia (VHS) in Slovenia. *Dis. Aquat. Org.*, **92**, 21–2.

UNITED STATES DEPARTMENT OF THE INTERIOR, US GEOLOGICAL SURVEY (2007). Detection of viral hemorrhagic septicemia virus. USGS FS 2007-3055. US Department of the Interior, US Geological Survey. Fact Sheets.

VESTERGARD JORGENSEN P.E. & OLESEN N.J. (1987). Cod ulcus syndrome rhabdovirus is indistinguishable from the Egtved (VHS) virus. *Bull. Eur. Ass. Fish Pathol.*, **7**, 73.

WALKER P.J., BENMANSOUR A., DIETZGEN R. *ET AL.* (2000). Family Rhabdoviridae. In: Virus Taxonomy. Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses, Van Regenmortel M.H.V., Fauquet C.M., Bishop D.H.L. *et al.*, eds. 563–583.

WARG J.V., CLEMENT T., CORNWELL E.R., CRUZ A., GETCHELL R.G., GIRAY C., GOODWIN A.E., GROOCCOCK G.H., FAISAL M., KIM R., MERRY G.E., PHELPS N.B.D., REISING M.M., STANDISH I., ZHANG Y. & TOOHEY-KURTH K. (2014a). Detection and surveillance of viral hemorrhagic septicemia virus using real-time RT-PCR. I. Initial comparison of four protocols. *Dis. Aquat. Org.*, **111**, 1–13.

WARG J.V., CLEMENT T., CORNWELL E.R., CRUZ A., GETCHELL R.G., GIRAY C., GOODWIN A.E., GROOCCOCK G.H., FAISAL M., KIM R., MERRY G.E., PHELPS N.B.D., REISING M.M., STANDISH I., ZHANG Y. & TOOHEY-KURTH K. (2014b). Detection and surveillance of viral hemorrhagic septicemia virus using real-time RT-PCR. II. Diagnostic evaluation of two protocols. *Dis. Aquat. Org.*, **111**, 15–22.

VERRIER E.R., DORSON M., MAUGER S., TORHY C., CIBOTARU C., HERVET C., DECHAMP N., GENET C., BOUDINOT P. & QUILLET E. (2013). Resistance to a Rhabdovirus (VHSV) in Rainbow Trout: Identification of a Major QTL Related to Innate Mechanisms. *PLoS ONE* **8**(2): e55302. doi:10.1371/journal.pone.0055302

WINTON J., KURATH G. & BATTS W. (2008). Molecular epidemiology of viral hemorrhagic septicemia virus in the Great Lakes region (USGS Fact Sheet 2008-3003). Available at <http://wfrc.usgs.gov/products/fs20083003.pdf>

UNOFFICIAL VERSION

Annex 14 (contd)

WOLF K. (1988). Viral hemorrhagic septicemia. *In*: Fish Viruses and Fish Viral Diseases. Cornell University Press, Ithaca, New York, USA, 217–249.

*
* *

NB: There are OIE Reference Laboratories for Infection with viral haemorrhagic septicaemia virus (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: <http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratories for any further information on infection with viral haemorrhagic septicaemia virus

NB: FIRST ADOPTED IN 1995 AS VIRAL HAEMORRHAGIC SEPTICAEMIA;
MOST RECENT UPDATES ADOPTED IN 2012.

UNOFFICIAL VERSION

CHAPTER 2.3.3.

INFECTION WITH *GYRODACTYLUS SALARIS***EU comment**

The EU in general supports the proposed changes to this chapter.

A couple of comments have been inserted within the text of this annex.

1. Scope

For the purpose of this chapter, *Gyrodactylus salaris* means infection with the pathogenic agent *G. salaris* (*G. salaris*) of the Genus *Gyrodactylus* and Family *Gyrodactylidae*, Order *Gyrodactylidea*, and Class *Monogenea*.

2. Disease information**2.1. Agent factors****2.1.1. Aetiological agent**

Several strains or clades of *G. salaris* have been identified on the basis of genotyping with the mitochondrial cytochrome oxidase 1 (CO1) marker (Hansen *et al.*, 2003; 2007b; Meinila *et al.*, 2002; 2004; Mieszkowska *et al.*, 2018). Although there does not seem to be an unambiguous correspondence between parasite strains as identified by CO1 and pathogenicity (Hansen *et al.*, 2007a), all strains recovered from Atlantic salmon (*Salmo salar*) that have been studied in laboratory experiments, so far, are highly pathogenic to strains of Atlantic salmon. Strains non-pathogenic to Atlantic salmon have been recovered from non-anadromous Arctic charr (*Salvelinus alpinus*) in Norway (Olstad *et al.*, 2007a; Robertsen *et al.*, 2007) and rainbow trout (*Oncorhynchus mykiss*) in Denmark (Jorgensen *et al.*, 2007; Lindenstrom *et al.*, 2003).

There has been a long taxonomic/scientific debate on whether *Gyrodactylus thymalli*, a species described from grayling (*Thymallus thymallus*), is a junior synonym of *G. salaris* (see e.g. Hansen *et al.*, 2003; 2007a, 2007b; Meinila *et al.*, 2004, Fromm *et al.*, 2014), and most evidence favours such a synonymization. The National Center for Biotechnology Information (NCBI) has accepted the synonymization of *G. salaris* and *G. thymalli* with the result that all accessions of DNA sequences previously assigned to *G. thymalli* are now assigned to *G. salaris*. Irrespective of this debate, strains isolated from grayling have never been found pathogenic to Atlantic salmon in experimental trials (see e.g. Sterud *et al.*, 2002), and do not seem to occur on Atlantic salmon when in sympatry with grayling (Anttila *et al.*, 2008). In this chapter, it is assumed that *G. salaris* and *G. thymalli* are two species.

2.1.2. Survival and stability on host tissues

Survival of *G. salaris* attached to a dead host is temperature dependent: maximum survival times for *G. salaris* on dead Atlantic salmon are 72, 142 and 365 hours at 18, 12 and 3°C, respectively (Olstad *et al.*, 2006).

2.1.3. Survival and stability off the host or in processed or stored samples

Survival of detached *G. salaris* is temperature dependent: approximately 24 hours at 19°C, 54 hours at 13°C, 96 hours at 7°C and 132 hours at 3°C (Olstad *et al.*, 2006). *G. salaris* is known to survive between temperatures of 0°C to 25°C. Tolerance to temperatures above 25°C is unknown. *G. salaris* is sensitive to freezing and desiccation. It dies after a few days at pH≤5. It is more sensitive to low pH (5.1<pH<6.4) in association with aluminium and zinc than the host Atlantic salmon (Poleo *et al.*, 2004; Soleng *et al.*, 1999), and recently, it was also found that *G. salaris* is sensitive to low doses of chlorine (Hagen *et al.*, 2014). For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with *G. salaris* according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Arctic char (*Salvelinus alpinus*), brown trout (*Salmo trutta*), grayling (*Thymallus thymallus*), and North American brook trout (*Salvelinus fontinalis*).

2.2.2. Species with incomplete evidence for susceptibility

None known.

2.2.3. Non-susceptible species

Species that have been found non-susceptible to infection with *G. salaris* according to Chapter 1.5. of the *Aquatic Code* are: [under study].

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

The prevalence and abundance of *G. salaris* on Atlantic salmon are higher than in other susceptible species. All life stages are susceptible, but prevalence and abundance in Atlantic salmon are highest in fry and parr stages, where mortality is also most likely to be observed.

2.2.5. Distribution of the pathogen on the host

G. salaris usually occurs on the fins of infected Atlantic salmon, but the parasite distribution on the host may vary depending on intensity of infection (Jensen & Johnsen, 1992; Mo, 1992; [Paladini *et al.*, 2014](#)). Parasites are also commonly found on the body but less commonly on the gills. On other hosts, the distribution may be different, but in general the parasite is relatively less abundant on the fins and relatively more common on the body compared with Atlantic salmon.

2.2.6. Aquatic animal reservoirs of infection

There are a number of combinations of host species and *G. salaris* strains which do not result in clinical signs of disease and may, therefore, act as reservoirs of infection. Some stocks of Atlantic salmon in the Baltic region are infected with *G. salaris* but do not generally show clinical signs or suffer mortality (Anttila *et al.*, 2008). *G. salaris* has been found in wild Arctic charr without any observable signs or mortality (Robertsen *et al.*, 2007). Rainbow trout can be infected with some strains of *G. salaris* at a very low prevalence and abundance without observable signs (Paladini *et al.*, 2014).

2.2.7. Vectors

G. salaris parasites may attach themselves to species not considered susceptible species, for short periods of time. Thus, any fish species could act as a vector, however, there is no evidence that they are important in the epidemiology of *G. salaris*.

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Mortality in farmed Atlantic salmon fry and parr can be 100% if not treated. Mortality in wild Atlantic salmon fry and parr in Norwegian rivers can be as high as 98%, with an average of about 85% (Johnsen *et al.*, 1999). Mortality in other susceptible species is usually low to negligible.

Prevalence in susceptible strains of Atlantic salmon reaches close to 100% in wild parr in rivers (Appleby & Mo, 1997); similarly, prevalence in farmed Atlantic salmon (in freshwater) rises to close to 100% within a short time after introduction of the parasite. Prevalence in resistant strains of Atlantic salmon in rivers and farms is unknown. Prevalence in other susceptible species is usually much lower than in Atlantic salmon and can be below 10% (e.g. in farmed rainbow trout; Buchmann & Bresciani, 1997).

2.3.2. Clinical signs, including behavioural changes

Usually there are no clinical signs in wild Atlantic salmon with infections of one or up to a few tens of *G. salaris* parasites. In the early disease phase in susceptible stocks of wild Atlantic salmon, increased

flashing (fish scratch their skin on the substrate) is typical. Later, fish may become greyish because of increased mucous production and the fins may be eroded. Diseased fish are lethargic and are usually found in slower-moving water.

Flashing is common among moderate to heavily infected farmed Atlantic salmon as they scratch their skin on the bottom or wall of a tank or pond. Heavily infected fish may have reduced activity and stay in low current areas.

Rainbow trout usually only carry low numbers of *G. salaris* parasites and do not show clinical signs.

2.3.3 Gross pathology

Heavily infected Atlantic salmon may become greyish as a result of increased mucification, and at a later stage the dorsal and pectoral fins may become whitish as a result of increased thickness (mainly hypertrophy) of the epidermis. As the infestation continues, fish may have eroded fins, especially dorsal, tail and pectoral fins, because of parasite feeding. Secondary fungal infections (*Saprolegnia* spp.) are commonly observed in fish with infection with *G. salaris*.

EU comment

In the above paragraph, the alteration of the fin is defined as ‘hypertrophy’ where the size of the cells increases. We consider that in this case the anatomopathological alteration is more a ‘hyperplasia’, where the cells increase their number but remain approximately with the same size. For this reason we suggest replacing ‘hypertrophy’ by ‘hyperplasia’.

2.3.4. Modes of transmission and life cycle

G. salaris is an obligate parasite with a direct life cycle. Parasites give birth to live offspring, and there are no other life stages. *G. salaris* can transfer to a new host via contact with live hosts, dead hosts, detached parasites drifting in the water column, or parasites attached to the substrate.

G. salaris has spread between rivers and farms mainly by the translocation of live fish. Fish migrating through brackish water can also spread the parasite between neighbouring rivers (see also Section 2.3.5). The risk of transmission is greater between rivers located within the same brackish water system.

2.3.5. Environmental and management factors

Although *G. salaris* mainly lives in fresh water, it reproduces normally at salinities up to 5–6 ppt. Survival at higher salinities is temperature dependent. For example at 1.4°C, *G. salaris* may survive for 240 hours, 78 hours and 42 hours at 10 ppt, 15 ppt and 20 ppt salinity, respectively, while at 12°C it may survive for 72 hours, 24 hours and 12 hours at the same three salinities, respectively (Soleng & Bakke, 1997).

G. salaris is sensitive to changes in the chemical composition of the water. It is sensitive to the most commonly used chemicals for bath treatment of farmed salmon parr and eggs (e.g. high salinity saltwater, formaldehyde and compounds containing chlorine and iodine). Furthermore, *G. salaris* is sensitive to acidic solutions (pH 5.0–6.0) of aluminium sulphate ($[Al_2(SO_4)_3]$) and zinc (ZN) (Poleo *et al.*, 2004; Soleng *et al.*, 1999). As aluminium sulphate is less toxic to fish than to *G. salaris* in moderately acidified waters, and this chemical has been used to eradicate the parasite from one river system in Norway (Pettersen *et al.*, 2007). *G. salaris* is sensitive to low doses of chlorine (Hagen *et al.*, 2014).

2.3.6. Geographical distribution

G. salaris is restricted in its distribution to Europe. It has been recovered from farmed Atlantic salmon or farmed rainbow trout in several (mainly northern) European countries. In the wild, the parasite has been found on wild salmonids, mainly Atlantic salmon parr, in rivers in Russia, Sweden, Finland and Norway. In some areas, the parasite continues to spread, and in 2015 it was detected on salmon parr in a new area in the north of Russia. In 2006, infection with *G. salaris* was reported from fish farms in Italy (Paladini *et al.*, 2009) and, in 2007, from fish farms in Poland (Rokicka *et al.*, 2007) and Macedonia (Zietara *et al.*, 2007). In 2009, *G. salaris* was identified from fish farms in Romania (Hansen *et al.*, 2014). The parasite has never been detected in the United Kingdom or in the Republic of Ireland.

For recent information on distribution at the country level consult the WAHIS interface (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en).

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

Vaccines are not available.

2.4.2. Chemotherapy including blocking agents

Not applicable.

2.4.3. Immunostimulation

Immunostimulation is not available.

2.4.4. Breeding resistant strains

In laboratory experiments, selected breeding of Atlantic salmon has resulted in increased survival among the offspring (Salte *et al.*, 2010). However, stocking rivers with resistant strains has not been attempted because the stock will remain infected and thus the parasite may spread to other rivers with susceptible hosts.

2.4.5. Inactivation methods

Not applicable.

2.4.6. Disinfection of eggs and larvae

Eggs that are transferred from infected farms should be disinfected (iodine-containing compounds have been used).

2.4.7. General husbandry

Treatment of farmed salmonid populations with formaldehyde or other bath treatments will reduce the prevalence and abundance of *G. salaris* and may therefore render detection more difficult.

Restocking with resistant strains of Atlantic salmon (e.g. Baltic Neva strain) in affected rivers is not considered compatible with existing strain management of Atlantic salmon (i.e. preservation of the genetic integrity of wild stocks) (Karlsson *et al.*, 2019).

The spread of *G. salaris* between freshwater fish farms and between rivers may be avoided by disinfection of equipment (e.g. fish nets) before translocation.

3. Specimen selection, sample collection, transportation and handling

This section draws on information in Sections 2.2, 2.3 and 2.4 to identify populations, individuals and samples that are most likely to be infected.

3.1. Selection of populations and individual specimens

Sampling wild healthy populations should take place during the late summer or autumn when prevalence is highest. Atlantic salmon should be targeted. In farms, fish showing clinical signs of infection (as described in Section 2.3.1) should be selected. Sampling should be avoided for a period after treatment for ectoparasites. In the absence of clinical signs sampling in wild populations should target year class 1+ and 2+ as these are more likely of being infected than 0+ parr.

EU comment

In the paragraph above and to take account of climatic variations between Member Countries, we suggest adding the following text at the end of the first sentence: ‘Sampling wild healthy populations should take place during the late summer or autumn or when the prevalence of the parasite is known to be at its highest’

3.2. Selection of organs or tissues

Detection of *Gyrodactylus* and identification of *G. salaris* is a two-step process. Firstly, gyrodactylid parasite specimens are detected (e.g. on fish or fins) using optical equipment and picked out, and individual parasites are identified to species level using other equipment and methods.

Fish should be examined as whole specimens either live under anaesthesia (for example, with MS222), freshly killed, or preserved. In addition, fresh or preserved fins can be examined. Examination of live, anaesthetised fish is very time-consuming and not recommended. When Atlantic strains of Atlantic salmon parr are infected, almost all fish have at least one *G. salaris* on one of the fins. On some fish, *G. salaris* specimens may occur on the body or head, including the nostrils, the gills and the mouth cavity. The distribution of *G. salaris* on fins and other parts of the fish varies among fish species and strains of Atlantic salmon. For all hosts the examination of whole fish is recommended as it will increase the likelihood of detecting low intensity infections.

Live anaesthetised fish, freshly cut fins or EtOH-preserved fish or fins should be examined under a binocular dissecting microscope with good illumination. The fish should be placed in a box and completely covered in fresh water. Preserved fish can also be examined in EtOH. Living parasites are more easily detected by their movements, thus disturbing light refraction on the skin of the fish should be avoided. Live *Gyrodactylus* are colourless while EtOH-preserved *Gyrodactylus* specimens are usually slightly opaque. Dark field illumination microscopy will increase the contrast and the parasites will be detected more easily. The whole surface of the fish, including gills and mouth cavity, must be examined. It is best to use two forceps for this process. The fins of relatively small fish, usually less than 10 cm, can also be studied using illumination through the bottom of the microscope stage, which makes *Gyrodactylus* specimens easy to observe.

3.3. Samples or tissues not suitable for pathogen detection

Dead fish, stored on ice, are not acceptable for *Gyrodactylus* examination, even if the fish are kept separately in plastic bags, etc. The parasites die quickly if not covered in water and rapidly disintegrate.

3.4. Non-lethal sampling

Fish can be examined as live specimens under anaesthesia (for example, with MS222). Recently, a non-lethal method for isolating specimens of gyrodactylid parasites from fish was developed and tested on brown trout (Thrush *et al.*, 2019). The method was shown to have a higher parasite recovery rate compared to whole body examination of killed fish (84.6% and 51.9%, respectively). The method has not yet been used on fish infected with *G. salaris* but it is likely to be effective.

In addition, environmental DNA (eDNA) methods for detection of *G. salaris* and its two main hosts, Atlantic salmon and rainbow trout, in water samples have been developed (Rusch *et al.*, 2018). However, detection limits have not been established for these analyses.

3.5. Preservation of samples for submission

Fish should be killed immediately and should not be allowed to dry out before preservation. Whole fish should be preserved in 80–100% EtOH in bottles large enough to provide excess space and preservative. The concentration of EtOH after preservation should not be below 70%. As a rule of thumb this concentration is obtained if the proportion of fish tissue to EtOH does not exceed 1:9. If the concentration is lower, the mucous and epidermis may disintegrate and *Gyrodactylus* specimens, even if they are preserved, may drop off. Bottles should have an opening wide enough to avoid the possibility of scraping off *Gyrodactylus* specimens when fish are put into the bottle or when taken out for examination. Bottles should be stored in a horizontal position until the tissue is fixed/preserved to prevent the fish curling. When preservation of the fish is complete, the bottles can be stored in a vertical position.

As *G. salaris* is common on fins of Atlantic salmon, fins cut off from the body and stored in EtOH as described above can also be submitted. This is especially suitable for larger fish and under field conditions where, for example, transport is limited.

Formaldehyde-fixed *Gyrodactylus* specimens are difficult to identify morphologically and are unsuitable for DNA analysis.

3.5.1. Samples for pathogen isolation

Not applicable.

3.5.2. Preservation of samples for molecular detection

Tissue samples, i.e. isolated parasites, whole fish or fins, for PCR testing should be preserved in 70–90% (v/v) analytical/reagent-grade (absolute) ethanol. The recommended ratio of ethanol to tissue is

9:1 based on studies in terrestrial animal and human health. The use of lower grade (laboratory or industrial grade) ethanol is not recommended.

Template DNA should be prepared from live/fresh or EtOH-preserved specimens using a suitable DNA preparation protocol. DNA extraction kits may be used according to the manufacturers' instructions.

3.5.3. Fixed samples for histopathology, immunohistochemistry or in-situ hybridisation

Not applicable.

3.5.4. Fixed samples for electron microscopy

Not applicable.

3.5.5. Samples for other tests

Preservation of samples for environmental DNA (eDNA) analyses

Several methods for filtering water for eDNA analyses exist and the method has also been developed for use on *G. salaris* and its hosts, *Salmo salar* and *Onchorhynchus mykiss* (Rusch *et al.*, 2018). Duplicate water samples of 5 litres (2 x 5 litres) should be collected and filtered on site on to glass fibre filters (47 mm AP25 Millipore, 2 µm pore size, Millipore, Billerica, USA) using a suitable pump, tubing and filter holder. Filters should be placed in separate zip-lock plastic bags containing silica gel and stored dry and dark until further analysis in the laboratory.

3.6. Pooling of samples

Sampled fish can be pooled, although each fish should subsequently be examined and analysed separately. Fins of fish from a farm or a river can be pooled and are also examined and analysed separately, but in this instance each fin cannot be related to a certain fish host. Material from parasites should not be pooled for molecular diagnostics.

4. Diagnostic methods

The methods currently available for identifying infection for surveillance (in healthy populations), presumptive and confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in the Table indicate:

Key:

- +++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway; OIE recommended method(s) will be mentioned in the text;
- ++ = Suitable method(s) but may need further validation;
- + = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;

Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on sensitivity, specificity, repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay sensitivity or specificity, such as tissue components inhibiting amplification, nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Morphological examination		+	+	1		+	+	1				
Histopathology ³												
Cytopathology ³												
Culture												
Real-time PCR (using parasite sample)		+	+	1		+	+	1				
ddPCR/Real-time PCR (using environmental sample)		+		1								
Conventional PCR										++	++	2
Amplicon sequencing ⁴										++	++	2
<i>In-situ</i> hybridisation												
Bioassay												
LAMP												
Ab-ELISA												
Ag-ELISA												

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); PCR = polymerase chain reaction; ddPCR = droplet digital PCR; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively;

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Early and juvenile life stages have been defined in Section 2.2.3.

³Histopathology and cytopathology can be validated if the results from different operators has been statistically compared. ⁴Sequencing of the PCR product.

Shading indicates the test is inappropriate or should not be used for this purpose

Annex 15 (contd)**4.1. Morphological examination**

Morphological identification of *Gyrodactylus* species is based on the morphology and morphometry of marginal hooks anchors (hamuli) and bars in the opisthaptor (the attachment organ). Good preparation of specimens is a prerequisite for species identification. Morphological identification is only recommended for preliminary diagnosis of *G. salaris* and should not be used for confirmation, for which molecular methods are recommended.

Digestion of the soft tissue, leaving the hard parts only, is recommended when high-resolution morphometrics is required for reliable morphometric diagnosis. The soft tissue can be digested in a solution (approx. 1 µl) of 75 mM Tris, 10 mM EDTA (ethylene diamine tetra-acetic acid), 5% SDS (sodium dodecyl sulphate) and 100 mg ml⁻¹ proteinase K, pH 8.0. After adding the digestion solution, the reaction should be inspected in the microscope until completion and then ended by adding a stop solution (1:1 glycerol and 10% neutral buffered formalin). The procedure for digestion is described in detail in Harris *et al.*, 1999. Identification of *G. salaris* should be in accordance with references: Cunningham *et al.*, 2001; Malmberg, 1957; 1970; McHugh *et al.*, 2000; Olstad *et al.*, 2007b; Shinn *et al.*, 2004.

The size of the opisthaptor hard parts in *Gyrodactylus* varies extensively with, for example, temperature, whereas shape is more stable (see e.g. Mo, 1991a). The capability of linear measurements to capture morphology might therefore not always be sufficient for reliable diagnosis (Olstad *et al.*, 2007b).

Gyrodactylus salaris can be differentiated from *Gyrodactylus* species by trained morphologists on the basis of morphology but not from *G. thymalli* (Olstad *et al.*, 2007b). In addition, *G. salaris* is morphologically similar to *Gyrodactylus teuchis* from brown trout, Atlantic salmon, and rainbow trout, but can be differentiated by trained morphologists on the basis of the shape of the marginal hook sickle. *G. teuchis* has a longer and more constantly curved sickle blade (see Cunningham *et al.*, 2001).

4.2. Histopathology and cytopathology

Not applicable.

4.3. Cell or artificial media culture for isolation

Not applicable.

4.4. Nucleic acid amplification

For all molecular tests below DNA can be extracted using standard DNA extraction kits.

4.4.1. Real-time PCR

Both real-time PCR (Collins *et al.*, 2010) and digital droplet (dd) PCR (Rusch *et al.*, 2018) have been developed for *G. salaris*. Real-time PCR has not been widely applied for diagnostics of *G. salaris*, and ddPCR is developed for use in connection with eDNA-methods. Both these methods target the ribosomal internal transcribed spacers region (ITS) and have the same diagnostic limitations as described in Sections 4.5.1 and 4.5.2.

4.4.2. Conventional PCR

Analysis of the ribosomal RNA gene internal transcribed spacer region (ITS)

*For amplification of a 1300 base pair product of the ITS-region, covering ITS1, 5.8S, and ITS2, primers, such as 5'-TTT-CCG-TAG-GTG-AAC-CT-3' and 5'-TCC-TCC-GCT-TAG-TGA-TA-3', may be used. The cycling conditions for PCR are as follows, initial denaturation at 95°C for 5 minutes; 30 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; final extension at 72°C for 7 minutes (Cunningham, 1997). If partially degraded material is analysed or if the PCR above does not give a positive result, the ITS1 and ITS2 spacers can be amplified in two separate reactions using primer sets and PCR conditions described in Matejusová *et al.*, 2001. The amplification of ITS2 alone, using the primers 5'-CAT-CGG-TCT-CTC-GAA-CG-3' and 5'-TCC-TCC-GCT-TAG-TGA-TA-3' and using the same protocol as above is sufficient.*

The primers for amplification of ITS are not specific to *G. salaris* and will amplify all or most species of *Gyrodactylus*. Positive PCR products should thus be sequenced for species confirmation (Section 4.5).

Analysis of the mitochondrial cytochrome oxidase I (CO1) gene

For amplification of the CO1-gene, the primers 5'-ATA-TAG-ACG-ATT-TGT-TTT-CA-3' and 5'-ACA-GAT-TAC-TTG-GTA-TTA-CA-3' (Kuusela *et al.*, 2009) may be used to amplify the full-length gene (1600 base pairs) which is recommended. The primers 5'-TAA-TCG-GCG-GGT-TCG-GTA-A-3' and 5'-GAA-CCA-TGT-ATC-GTG-TAG-CA-3' (Meinila *et al.*, 2002) may be used to amplify a 800 base pairs fragment if the first PCR is unsuccessful. The cycling conditions for both PCRs are as follows, initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes; final extension at 72°C for 7 minutes. Additional primer sets for amplification of CO1 can be found in references: [Meinila *et al.*, 2002](#); [2004](#), [Kuusela *et al.*, 2009](#).

Primers recommended for amplification of CO1 might not be specific for *G. salaris*. Positive PCR products should thus be sequenced for species confirmation (Section 4.5).

The following controls should be run with each assay: negative extraction control; positive control; no template control.

4.4.3. Other nucleic acid amplification methods

Not applicable.

4.5. Amplicon sequencing

4.5.1. ITS sequencing and sequence analysis

Amplified ITS fragments prepared as in Section 4.4.2 should be sequenced using the PCR primers and, in addition, internal sequencing primers (Cunningham, 1997; Matejusová *et al.*, 2001) should be used to obtain overlapping reads of each nucleotide. The resulting ITS sequences should be subjected to a BLAST search in GenBank/EMBL to establish identity with known sequences. Several sequences of other species infecting salmonids, e.g. *G. derjavini*, *G. derjavinoidea*, *G. truttae*, and *G. teuchis* are available in GenBank/EMBL. *G. thymalli* cannot be distinguished from *G. salaris* by this method, but sequences of ITS distinguishes *G. salaris* from all other known species. If the BLAST search identifies the parasite as *G. salaris*, CO1 sequencing and sequence analysis should be performed (Section 4.5.2).

4.5.2. CO1 sequencing and sequence analysis

Amplified CO1 fragments prepared as described in Section 4.4.2 should be sequenced and, in addition, internal sequencing primers (Kuusela *et al.*, 2009; Meinila *et al.*, 2002) should be used to obtain overlapping reads of each nucleotide. The resulting CO1 sequences should be subjected to a BLAST search in GenBank/EMBL to identify the haplotype.

If the obtained sequence does not have a 100% match in GenBank/EMBL, a phylogenetic analysis can be performed to establish the relationship to other available sequences. Different haplotypes and clades of *G. salaris* and *G. thymalli* can be distinguished with this method. CO1 sequences can be used to assign specimens to a haplotype or clade and thus infer the identity as *G. salaris* or *G. thymalli*. Clades (haplogroups) of *G. salaris* generally correspond well to host preferences and/or the geographical distribution of the parasites, with a few exceptions, and some strains, as defined by CO1-sequences (haplotypes), are known to be pathogenic to Atlantic salmon. Host identity can be used to infer potential pathogenicity of a certain strain and thus host identity of sequence hits in GenBank/EMBL should always be checked when BLAST results are returned.

Where the sequence is not assigned to one of the recognised haplotypes (CO1 sequences) of *G. salaris* or *G. thymalli* advice should be sought from the OIE Reference Laboratory. The OIE Reference laboratory will keep an updated database of CO1-sequences and will assist in the diagnosis. It is recommended that the OIE Reference laboratory is informed of any significant detections of *G. salaris* and *G. thymalli* in order to confirm the cases.

4.6. In-situ hybridisation

Not applicable.

4.7. Immunohistochemistry

Not applicable.

Annex 15 (contd)**4.8. Bioassay**

Not applicable.

4.9. Antibody- or antigen-based detection methods (ELISA, etc.)

Not applicable.

4.10. Other methods

Not applicable.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

Real-time PCR is the recommended test for surveillance to demonstrate freedom of disease in apparently healthy population. Sequencing of the amplified CO1 amplicon is required for confirmation of infection in any parasite that identified as positive PCR

6. Corroborative diagnostic criteria

All suspect positive samples of *G. salaris* from country or zone or compartment considered free from infection with *G. salaris* should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case.

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1) or presence of clinical signs (Section 6.2) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent.

6.1. Apparently healthy animals or animals of unknown health status⁵

Healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations will be sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with *G. salaris* shall be suspected if at least one of the following criteria is met:

- i) Identification of *G. salaris* by morphological examination
- ii) A positive result by real-time PCR
- iii) A positive result by ddPCR or real-time-PCR from an environmental sample.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with *G. salaris* is considered to be confirmed if, in addition to the criteria in Section 6.1.1., the following criterion is met:

- i) A positive result from sequencing amplified CO1 fragments obtained by conventional PCR.

6.2 Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with *G. salaris* shall be suspected if at least one of the following criteria is met:

⁵ For example, transboundary commodities.

- i) Gross pathology or clinical signs associated with the disease as described in this chapter, with or without elevated mortality
- ii) Identification of *G. salaris* by morphological examination
- iii) A positive result by real-time PCR.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with *G. salaris* is considered to be confirmed if, in addition to the criteria in section 6.2.1. the following criterion is met:

- i) A positive result from sequencing of amplified CO1 fragments obtained by conventional PCR.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with *G. salaris* are provided in Table 6.3. (note: no data are currently available). This information can be used for the design of surveys for infection with *G. salaris*, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

Table 6.3. Diagnostic performance of tests recommended for surveillance or diagnosis

Test type	Test purpose	Source population	Tissue/ sample type	Species	DSe (n)	DSp (n)	Reference test	Citation
Real-time PCR	Surveillance	–	Parasites	–	Not yet available	Not yet available	–	–
Amplicon sequencing	Diagnosis	–	Parasites	–	Not yet available	Not yet available	–	–

DSe = diagnostic sensitivity; DSp = diagnostic specificity; n = number of samples used in the study;

7. References

- ANTTILA P., ROMAkkANIEMI A., KUUSELA J. & KOSKI P. (2008). Epidemiology of *G. salaris* (Monogenea) in the River Tornionjoki, a Baltic wild salmon river. *J. Fish Dis.*, **31**, 373–382.
- APPLEBY C. & MO T.A. (1997). Population Dynamics of *G. salaris* (Monogenea) Infecting Atlantic Salmon, *Salmo salar*, Parr in the River Batnfjordselva, Norway. *J. Parasitol.*, **83**, 23–30
- BUCHMANN K. & BRESCIANI J. (1997). Parasitic infections in pond-reared rainbow trout *Oncorhynchus mykiss* in Denmark. *Dis. Aquat. Org.*, **28**, 125–138.
- COLLINS C.M., KERR R., MCINTOSH R. & SNOW M. (2010). Development of a real-time PCR assay for the identification of *Gyrodactylus* parasites infecting salmonids in northern Europe. *Dis. Aquat. Org.*, **90**, 135–142.
- CUNNINGHAM C.O. (1997). Species variation within the internal transcribed spacer (ITS) region of *Gyrodactylus* (Monogenea: Gyrodactylidae) ribosomal RNA genes. *J. Parasitol.*, **83**, 215–219.
- CUNNINGHAM C.O., MO T.A., COLLINS C.M., BUCHMANN K., THIERY R., BLANC G. & LAUTRAITE A. (2001). Redescription of *Gyrodactylus teuchis* Lautraite, Blanc, Thiery, Daniel & Vigneulle, 1999 (Monogenea: Gyrodactylidae), a species identified by ribosomal RNA sequence. *Syst. Parasitol.*, **48**, 141–150.
- HAGEN A.G., HYTTERØD S. & OLSTAD K. (2014). Low concentrations of sodium hypochlorite affect population dynamics in *Gyrodactylus salaris* (Malmberg, 1957): practical guidelines for the treatment of the Atlantic salmon, *Salmo salar* L. parasite. *J. Fish Dis.*, **37**, 1003–1011. <https://doi.org/10.1111/jfd.12218>
- HANSEN H., BACHMANN L. & BAKKE T.A. (2003). Mitochondrial DNA variation of *Gyrodactylus* spp. (Monogenea, Gyrodactylidae) populations infecting Atlantic salmon, grayling, and rainbow trout in Norway and Sweden. *Int. J. Parasitol.*, **33**, 1471–1478.

Annex 15 (contd)

- HANSEN H., BAKKE T.A. & BACHMANN L. (2007a). DNA taxonomy and barcoding of monogenean parasites: lessons from *Gyrodactylus*. *Trends Parasitol.*, **23**, 363–367.
- HANSEN H., BAKKE T.A. & BACHMANN L. (2007b). Mitochondrial haplotype diversity of *Gyrodactylus thymalli* (Platyhelminthes; Monogenea): extended geographic sampling in United Kingdom, Poland, and Norway reveals further lineages. *Parasitol. Res.*, **100**, 1389–1394.
- HARRIS P.D., CABLE J., TINSLEY R.C. & LAZARUS C.M. (1999). Combined ribosomal DNA and morphological analysis of individual gyrodactylid monogeneans. *J. Parasitol.*, **85**, 188–191.
- JENSEN A.J. & JOHNSEN B.O. (1992). Site Specificity of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea) on Atlantic Salmon (*Salmo salar* L.) in the River Lakselva, northern Norway. *Can. J. Zool.*, **41**, 264–267.
- JOHNSEN B.O., MØKKELGJERD P. & JENSEN A.J. (1999). The parasite *Gyrodactylus salaris* on salmon parr in Norwegian rivers, status report at the beginning of year 2000. In: NINA Oppdragsmelding, p. 1–129 (In Norwegian with English summary).
- JØRGENSEN T.R., LARSEN T.B., JØRGENSEN L.G., BRESCIANI J., KANIA P. & BUCHMANN K. (2007). Characterisation of a low pathogenic strain of *Gyrodactylus salaris* from rainbow trout. *Dis. Aquat. Org.*, **73**, 235–244.
- KARLSSON S., BOLSTAD G.H., HANSEN H., JANSEN P.A., MOEN T. & NOBLE LR. (2019). The potential for evolution of resistance to *Gyrodactylus salaris* in Norwegian Atlantic salmon. Norwegian Institute for Nature Research (NINA) Report 1812. Norwegian Institute for Nature Research. ISSN: 1504-3312 ISBN: 978-82-426-4570-8.
- KUUSELA J., ZIETARA M.S. & LUMME J. (2009). Description of three new European cryptic species of *Gyrodactylus* Nordmann, 1832 supported by nuclear and mitochondrial phylogenetic characterization. *Acta Parasitol.*, **53**, 120–126.
- LINDENSTROM T., COLLINS C.M., BRESCIANI J., CUNNINGHAM C.O. & BUCHMANN K. (2003). Characterization of a *Gyrodactylus salaris* variant: infection biology, morphology and molecular genetics. *Parasitology*, **127**, 165–177.
- MALMBERG G. (1957). Om förekomsten av *Gyrodactylus* på svenska fiskar. *Skr. söd. Sver. Fisk För.*, (Årsskr.) 1956, 19–76. (In Swedish, species descriptions and summary in English).
- MALMBERG G. (1970). The excretory systems and the marginal hooks as a basis for the systematics of *Gyrodactylus* (Trematoda, Monogenea). *Ark. Zool. [Ser. 2]*, **23**, 1–235.
- MATEJUSOVÁ I., GELNAR M., McBEATH A.J.A., COLLINS C.M. & CUNNINGHAM C.O. (2001). Molecular markers for gyrodactylids (Gyrodactylidae: Monogenea) from five fish families (Teleostei). *Int. J. Parasitol.*, **31**, 738–745.
- McHUGH E.S., SHINN A.P. & KAY J.W. (2000). Discrimination of the notifiable pathogen *Gyrodactylus salaris* from *G. thymalli* (Monogenea) using statistical classifiers applied to morphometric data. *Parasitology*, **121**, 315–323.
- MEINILA M., KUUSELA J., ZIETARA M. & LUMME J. (2002) Primers for amplifying approximately 820 bp of highly polymorphic mitochondrial COI gene of *Gyrodactylus salaris*. *Hereditas*, **137**, 72–74.
- MEINILA M., KUUSELA J., ZIETARA M.S. & LUMME J. (2004). Initial steps of speciation by geographic isolation and host switch in salmonid pathogen *Gyrodactylus salaris* (Monogenea: Gyrodactylidae). *Int. J. Parasitol.*, **34**, 515–526.
- MIESZKOWSKA A., GORNIK M., JURCZAK-KUREK A. & ZIETARA M.S. (2018). Revision of *Gyrodactylus salaris* phylogeny inspired by new evidence for Eemian crossing between lineages living on grayling in Baltic and White sea basins. *PeerJ.*, 6:e5167. doi:10.7717/peerj.5167
- MO T.A. (1992). Seasonal variations in the prevalence and infestation intensity of *Gyrodactylus salaris* Malmberg, 1957 (Monogenea: Gyrodactylidae) on Atlantic salmon parr, *Salmo salar* L., in the River Batnfjordselva, Norway. *J. Fish Biol.*, **41**, 697–707.
- OLSTAD K., CABLE J., ROBERTSEN G. & BAKKE T. A. (2006). Unpredicted transmission strategy of *Gyrodactylus salaris* (Monogenea: Gyrodactylidae): survival and infectivity of parasites on dead hosts. *Parasitology*, **133**, 33–41.
- OLSTAD K., ROBERTSEN G., BACHMANN L. & BAKKE T.A. (2007a). Variation in host preference within *Gyrodactylus salaris* (Monogenea): an experimental approach. *Parasitology*, **134**, 589–597.

Annex 15 (contd)

- OLSTAD K., SHINN A.P., BACHMANN L. & BAKKE T.A. (2007b). Host-based identification is not supported by morphometrics in natural populations of *Gyrodactylus salaris* and *G. thymalli* (Platyhelminthes, Monogenea). *Parasitology*, **134**, 2041–2052.
- PALADINI G., GUSTINELLI A., FIORAVANTI M.L., HANSEN H. & SHINN A.P. (2009). The first report of *Gyrodactylus salaris* Malmberg 1957 (Platyhelminthes, Monogenea) on Italian cultured stocks of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Vet. Parasitol.*, **165**, 290–297.
- PALADINI G., HANSEN H., WILLIAMS C.F., TAYLOR N.G.H., RUBIO-MEJÍA O.L., DENHOLM S., HYTTERØD S., BRON J.E. & SHINN A.P. (2014). Reservoir hosts for *Gyrodactylus salaris* may play a more significant role in epidemics than previously thought. *Parasit. Vectors*, **7**, 576.
- PETTERSEN R.T., HYTTERØD S., MO T.A., HAGEN A.G., FLODMARK L.E.W., HØGBERGET R., OLSEN N., KJØSNES A.J., ØXNEVAD S., HÅVARDSTUN J., KRISTENSEN T., SANDODDEN R., MOEN A. & LYDERSEN E. (2007). Kjemisk behandling mot *G. salaris* i Lærdalselva 2005/2006 – Sluttrapport. NIVA-rapport 5349-2007. 27 pp. (in Norwegian).
- POLEO A.B.S., SCHJOLDEN J., HANSEN H., BAKKE T.A., MO T.A., ROSSELAND B.O. & LYDERSEN E. (2004). The effect of various metals on *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). *Parasitology*, **128**, 169–177.
- ROBERTSEN G., HANSEN H., BACHMANN L. & BAKKE T. A. (2007). Arctic charr (*Salvelinus alpinus*) is a suitable host for *Gyrodactylus salaris* (Monogenea, Gyrodactylidae) in Norway. *Parasitology*, **134**, 257–267.
- ROKICKA M., LUMME J. & ZIĘTARA M. (2007). Identification of *Gyrodactylus* ectoparasites in Polish salmonid farms by PCR-RFLP of the nuclear ITS segment of ribosomal DNA (Monogenea, Gyrodactylidae). *Acta Parasitologica*, **52**, 185–195.
- RUSCH J.C., HANSEN H., STRAND D.A., MARKUSSEN T., HYTTERØD S. & VRÅLSTAD T. (2018). Catching the fish with the worm: a case study on eDNA detection of the monogenean parasite *G. salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Parasit. Vectors*, **11**, 333. doi:10.1186/s13071-018-2916-3
- SALTE R., BENTSEN H.B., MOEN T., TRIPATHY S., BAKKE T.A., ØDEGÅRD J., OMHOLT S. HANSEN L.P. (2010). Prospects for a genetic management strategy to control *Gyrodactylus salaris* infection in wild Atlantic salmon (*Salmo salar*) stocks. *Can. J. Fish. Aquat. Sci.*, **67**, 121–129.
- SHINN A.P., HANSEN H., OLSTAD K., BACHMANN L. & BAKKE T.A. (2004). The use of morphometric characters to discriminate specimens of laboratory-reared and wild populations of *Gyrodactylus salaris* and *G. thymalli* (Monogenea). *Folia Parasitol.*, **51**, 239–252.
- SOLENG A. & BAKKE T.A. (1997). Salinity tolerance of *Gyrodactylus salaris* (Platyhelminthes, Monogenea): laboratory studies. *Can. J. Fish. Aquat. Sci.*, **55**, 1837–1845.
- SOLENG A., POLEO A.B.S., ALSTAD N.E.W. & BAKKE T. A. (1999). Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. *Parasitology*, **119**, 19–25.
- STERUD E., MO T.A., COLLINS C. M. & CUNNINGHAM C.O. (2002). The use of host specificity, pathogenicity, and molecular markers to differentiate between *G. salaris* Malmberg, 1957 and *G. thymalli* Žitňan, 1960 (Monogenea: Gyrodactylidae). *Parasitology*, **124**, 203–213.
- THRUSH M.A., HILL T. & TAYLOR N.G.H. (2019). Development of a non-lethal hydrogen peroxide treatment for surveillance of *Gyrodactylus salaris* on trout farms and its application to testing wild salmon populations. *Transbound. Emerg. Dis.*, doi:10.1111/tbed.13263.

Annex 15 (contd)

ZIETARA M.S., ROKICKA, M., STOJANOVSKI S., SKORKOWSKI E.F. & LUMME J. (2007). Alien mitochondrial DNA in variant clones of *Gyrodactylus salaris* indicates a complex hybrid history in salmonid farms. 7th International Symposium on Fish Parasites, Viterbo, Italy. *Parassitologia*, **49**, 119.

*
* *

NB: There is an OIE Reference Laboratory for infection with *G. salaris*
(see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list:
<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).
Please contact the OIE Reference Laboratories for any further information on infection with *G. salaris*.

NB: FIRST ADOPTED IN 1997 AS GYRODACTYLOSIS OF ATLANTIC SALMON (*GYRODACTYLUS* F);
MOST RECENT UPDATES ADOPTED IN 2018.

UNOFFICIAL VERSION

CHAPTER 2.3.6.

INFECTION WITH SALMONID ALPHAVIRUS

EU comment

The EU supports the proposed changes to this chapter.

1. Scope

Infection with salmonid alphavirus (SAV) means infection with any genotype of the pathogenic agent SAV, Genus *Alphavirus* and Family *Togaviridae*.

2. Disease information**2.1. Agent factors****2.1.1. Aetiological agent**

SAV is an enveloped, spherical, single-stranded, positive-sense RNA virus, approximately 60–70 nm in diameter, with a genome of ~12 kb. The genome codes for eight proteins: four capsid glycoproteins (E1, E2, E3 and 6K) and four nonstructural proteins (nsP1–4). Glycoprotein E2 is considered to be the site of most neutralising epitopes, while E1 contains more conserved, cross-reactive epitopes (McLoughlin & Graham, 2007). SAV is considered to belong to the Genus *Alphavirus* of the Family *Togaviridae*. This is based on nucleotide sequence studies of SAV isolates, and is also supported by biological properties of the virus, including cross-infection and neutralisation trials. In addition, four conserved nucleotide sequence elements (CSEs) and a conserved motif (GDD), characteristic of alphaviruses, are present in the SAV genome (McLoughlin & Graham, 2007).

SAV has been divided into six genotypes (SAV 1–SAV 6) based solely on nucleic acid sequences for the proteins E2 and nsP3 (Fringuelli *et al.*, 2008). The level of antigenic variation among genotypes is considered low as monoclonal antibodies (MAbs) raised against a specific SAV genotype are likely to cross react with other SAV isolates (Graham *et al.*, 2014; Jewhurst *et al.*, 2004). The genotype groups by susceptible species and environment are presented in Table 2.1.

Infection with SAV causes pancreas disease (PD) or sleeping disease (SD) in Atlantic salmon (*Salmo salar* L.), common dab (*Limanda limanda*), rainbow trout (*Oncorhynchus mykiss*) (McLoughlin & Graham, 2007) and Arctic charr (*Salvelinus alpinus*) (Lewisch *et al.*, 2018). The disease is systemic, characterised microscopically by necrosis and loss of exocrine pancreatic tissue, and heart and skeletal muscle necrosis and atrophy.

Table 2.1. SAV genotypes by susceptible host species and environment

SAV genotype	Freshwater	Sea water
SAV 1	Rainbow trout	Atlantic salmon
SAV 2	Rainbow trout; Atlantic salmon; Arctic charr	Atlantic salmon
SAV 3		Rainbow trout; Atlantic salmon
SAV 4		Atlantic salmon
SAV 5		Atlantic salmon; Common dab
SAV 6		Atlantic salmon

2.1.2. Survival and stability in processed or stored samples

There are no published scientific data specifically on the survival and stability of SAV in processed or stored samples. The OIE Reference Laboratory has found that SAV in serum/plasma samples and virus isolated from cell culture can be stored for many years at -80°C without significant decline in virus titre. This observation is consistent with research on other alphaviruses.

2.1.3. Survival and stability outside the host

Laboratory tests suggest that SAV would survive for extended periods in the aquatic environment. In these tests, virus survival was inversely related to temperature. In the presence of organic matter, markedly longer survival times were observed in sea water compared with fresh water (Graham *et al.*, 2007b).

The half-life of SAV in serum has been found to be inversely related to temperature, emphasising the need for rapid shipment of samples at 4°C to laboratories for virus isolation. For long-term conservation of SAV-positive samples and cultured virus, storage at -80°C is recommended (Graham *et al.*, 2007b).

For inactivation methods, see Section 2.4.5.

2.2. Host factors

2.2.1. Susceptible host species

Species that fulfil the criteria for listing as susceptible to infection with SAV according to Chapter 1.5. of the *Aquatic Animal Health Code (Aquatic Code)* include: Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*), common dab (*Limanda limanda*) and rainbow trout (*Oncorhynchus mykiss*).

2.2.2. Species with incomplete evidence for susceptibility

Species for which there is incomplete evidence for susceptibility according to Chapter 1.5. of the *Aquatic Code* include: long rough dab (*Hippoglossoides platessoides*), plaice (*Pleuronectes platessa*) and Ballan wrasse (*Labrus bergylta*).

In addition, pathogen-specific positive polymerase chain reaction (PCR) results have been reported in the following species, but an active infection has not been demonstrated: Argentine hake (*Merluccius hubbsi*), brown trout (*Salmo trutta*), cod (*Gadus morhua*), European flounder (*Platichthys flesus*), haddock (*Melanogrammus aeglefinus*), herring (*Clupea harengus*), Norway pout (*Trisopterus esmarkii*), saithe (*Pollachius virens*), longhorn sculpin (*Myoxocephalus octodecemspinosus*) and whiting (*Merlangius merlangus*).

2.2.3. Non-susceptible species

Species that have been found non-susceptible to infection with SVCV according to Chapter 1.5. of the *Aquatic Code* are: No species are listed as non-susceptible.

2.2.4. Likelihood of infection by species, host life stage, population or sub-populations

Farmed Atlantic salmon and rainbow trout are the species with the highest likelihood of infection with SAV. Experimental studies have demonstrated that all life stages are susceptible to infection (Taksdal & Sindre, 2016). SAV 1–SAV 6 have been detected in Atlantic salmon. SAV 2 and SAV 3 have been detected in rainbow trout.

2.2.5. Distribution of the pathogen in the host

The heart and the pancreas are main target organs for infection with SAV. Necrosis and loss of exocrine pancreatic tissue, myocarditis and skeletal myositis are typical histopathological findings. During the viraemic stage, substantial amounts of virus are also found in serum, and during the infection virus can also be found in kidney, spleen, gills, mucous and faeces (Taksdal & Sindre, 2016).

2.2.6. Aquatic animal reservoirs of infection

There is evidence that some survivors of outbreaks will become long-term carriers of the virus (Graham *et al.*, 2009) and thus farmed Atlantic salmon and rainbow trout can be considered the main reservoir of SAV (Taksdal & Sindre, 2016). Infection with SAV has been detected in some wild flatfish species in Scotland (Bruno *et al.*, 2014; Snow *et al.*, 2010) which could also act as a reservoir of infection.

2.2.7. Vectors

Although most alphaviruses are transmitted by arthropod vectors, vector transmission of SAV has not yet been demonstrated. SAV has been detected by reverse-transcription (RT) PCR in salmon lice (*Lepeophtheirus salmonis*) collected during acute outbreaks of pancreas disease in Atlantic salmon, but transfer to susceptible fish species has not been reported (Pettersen *et al.*, 2009).

2.3. Disease pattern

2.3.1. Mortality, morbidity and prevalence

Mortality rates due to infection with SAV may vary with genotype, season, year, use of biosecurity measures and species of fish (Bang Jensen *et al.*, 2012; Graham *et al.*, 2011; Rodger & Mitchell, 2007; Stormoen *et al.*, 2013). The cumulative mortality at the farm level ranges from negligible to over 50% in severe cases (Bang Jensen *et al.*, 2012; Graham *et al.*, 2003; Rodger & Mitchell, 2007; Ruane *et al.*, 2008; Stene *et al.*, 2014). Experimental studies have demonstrated that SAV 2 infection in marine fish causes lower mortality than SAV 3 (Taksdal *et al.*, 2015).

Duration of disease outbreaks, defined as the period with increased mortality, may vary from 1 to 32 weeks (Jansen *et al.* 2010a; 2014; Ruane *et al.*, 2008).

The prevalence of infection with SAV may vary. During disease outbreaks, the prevalence is usually high; prevalences of 70–100% have been reported in Atlantic salmon farming sites (Graham *et al.*, 2010).

Prevalences in wild fish are largely unknown. SAV has been detected by PCR in some marine flatfish species in Scottish waters (Snow *et al.*, 2010). A serological survey of wild salmonids in freshwater river systems in Northern Ireland did not detect virus neutralisation antibodies against SAV in any of 188 sera tested, whereas the majority of sera from farmed salmon in sea water in the same area tested positive (Graham *et al.*, 2003).

2.3.2. Clinical signs, including behavioural changes

A sudden drop in appetite may be observed 1–2 weeks before the detection of elevated mortality. Clinically diseased fish may be observed swimming slowly at the water surface. In some cases, extremely weak (“sleeping”) fish can be found at the bottom of tanks or in net-cages. An increased number of faecal casts may also be observed. However, it is important to note that clinical signs are not pathognomonic.

Initially, nutritional status is usually normal, but in the months after an outbreak or in the later stages of disease, long slender fish (‘runts’) with poor body condition are typically observed. However, the presentation of long, slender fish can be caused by factors other than SAV.

2.3.3 Gross pathology

Yellow mucoid gut contents is a usual post-mortem finding, typically seen in inappetent fish. Occasionally, signs of circulatory disturbances, such as petechial haemorrhages, small ascites or reddening of the pancreatic region between the pyloric caeca, may be seen. Some diseased fish may show pale hearts or heart ruptures. It is important to note that post-mortem findings are not pathognomonic.

2.3.4. Modes of transmission and life cycle

Horizontal transmission of SAV is demonstrated by a range of evidence including: phylogenetic studies, successful transmission among cohabiting fish, proven transmission between farming sites, studies on survival of SAV in sea water and the spread via water currents (Graham *et al.*, 2007b; 2011; Jansen *et al.*, 2010a; Kristoffersen *et al.*, 2009; Viljugrein *et al.*, 2009).

Long-distance transmission, and thus introduction of SAV into a previously uninfected area, is most likely due to movement of infected live fish (Kristoffersen *et al.*, 2009; Rodger & Mitchell, 2007). SAV has been detected in fat leaking from dead fish which accumulates at the sea water surface, contributing to long distance spread of the virus (Stene *et al.*, 2013). Once SAV has been introduced into an area, farm proximity and water currents influence local transmission (Aldrin *et al.*, 2010; Kristoffersen *et al.*, 2009; Viljugrein *et al.*, 2009).

Vertical transmission of SAV has been suggested (Bratland & Nylund, 2009), but not demonstrated (Kongtorp *et al.*, 2010; McLoughlin & Graham, 2007). The Norwegian Scientific Committee for Food Safety, (2010), carried out a risk assessment and concluded that the risk of vertical transmission of SAV is negligible.

2.3.5. Environmental and management factors

Clinical outbreaks and mortality are influenced by water temperature and season (McLoughlin & Graham, 2007; Rodger & Mitchell, 2007; Stene *et al.*, 2014; Stormoen *et al.*, 2013). Stressing the fish by movement, crowding or treatment may initiate disease outbreaks on infected farms.

Annex 16 (contd)

Risk factors for outbreaks on a farming site include a previous history of infection with SAV, high feeding rate, high sea lice burden, the use of autumn smolts and previous outbreaks of infectious pancreatic necrosis (IPN) (Bang Jensen *et al.*, 2012; Kristoffersen *et al.*, 2009; Rodger & Mitchell, 2007).

2.3.6. Geographical distribution

Infection with SAV has been reported from several countries in Europe. See WAHIS (https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home/index/newlang/en) for recent information on distribution at the country level.

2.4. Biosecurity and disease control strategies

2.4.1. Vaccination

DNA-based and virus-inactivated vaccines against SAV are both commercially available. The vaccines may cause a risk of false positives, both in serological and PCR-based tests, according to data presented by vaccine companies. However, reports from the field indicates that false positives to serological tests do not occur after sea transfer. To prevent false positives by RT-PCR, sampling from vaccinated individuals should use heart tissue to avoid opening the abdominal cavity.

2.4.2. Chemotherapy including blocking agents

No chemotherapy is available.

2.4.3. Immunostimulation

No immunostimulation is available.

2.4.4. Breeding resistant strains

Differences in susceptibility among different family groups of Atlantic salmon have been observed in challenge experiments and in the field, indicating the potential for breeding for resistance (Norris *et al.*, 2008; Gonen *et al.*, 2015). Breeding programmes in Ireland and Norway have successfully produced fish with increased resistance to disease caused by SAV, which are now commercially available.

2.4.5. Inactivation methods

SAV is rapidly inactivated in the presence of high levels of organic matter at 60°C, pH 7.2, and at 4°C, pH 4 and pH 12, suggesting that composting, ensiling and alkaline hydrolysis would all be effective at inactivating virus in fish waste (Graham *et al.*, 2007a). The virus is also readily inactivated by UV-light, but is more resistant to chlorine and ozone treatment.

2.4.6. Disinfection of eggs and larvae

Standard disinfection procedures are considered sufficient to prevent surface contamination of eggs by SAV (Graham *et al.*, 2007).

2.4.7. General husbandry

To avoid infection with SAV, good husbandry practices should be applied such as use of appropriate sites for farming, segregation of generations, stocking with good quality fish, removal of dead fish, regular cleaning of tanks and pens, control of parasites and other pathogens, as well as careful handling of fish. Once an outbreak has started, mortality may be reduced by minimising handling and ceasing feeding.

3. Specimen selection, sample collection, transportation and handling

3.1. Selection of populations and individual specimens

Clinical inspections should be carried out during a period when the water temperature is below XX°C. All production units (ponds, tanks, net-cages, etc.) should be inspected for the presence of dead, weak or abnormally behaving fish. Extremely weak ('sleeping') fish may be found at the bottom of a tank or in the net-cages. If the number of clinically diseased fish is low, samples from long, thin fish ('runts') may be added (Jansen *et al.*, 2010b). If moribund or thin fish or runts are sampled, the probability of detecting SAV

is higher than if randomly selected, apparently healthy fish are sampled (Jansen *et al.*, 2010b). Prevalence estimates will also vary with the diagnostic method used.

UNOFFICIAL VERSION

Fish to be sampled are selected as follows:

- i) Susceptible species should be sampled proportionally, or following risk-based criteria for targeted selection of lots or populations with a history of abnormal mortality or potential exposure events (e.g. via untreated surface water, wild harvest or replacement with stocks of unknown disease status).
- ii) If more than one water source is used for fish production, fish from all water sources should be included in the sample.
- iii) If weak, abnormally behaving or freshly dead (not decomposed) fish are present, such fish should be selected. If such fish are not present, the fish selected should include normal appearing, healthy fish collected in such a way that all parts of the farm as well as all year classes are proportionally represented in the sample.

3.2. Selection of organs or tissues

Heart and mid-kidney are the recommended organs for detection of SAV either by molecular biological methods or by cell culture. During the course of the disease, the heart usually contains more SAV than other tissues and should always be sampled. After disease outbreaks, gill and heart tissue (Graham *et al.*, 2010) and pools of heart and mid-kidney tissue (Jansen *et al.*, 2010b) remained positive by real time RT-PCR for months after initial detection.

For sampling from vaccinated fish, the heart should be sampled without opening the abdominal cavity. Sampling of mid-kidney, spleen or other internal organs is not recommended to avoid contamination of viral RNA/DNA from the vaccine (See Section 2.4).

During the initial viraemic phase, serum samples are also suitable for detection of SAV either by molecular biological methods or by cell culture, which can provide an early warning of disease outbreaks (Graham *et al.*, 2010). From approximately 3 weeks after SAV infection, blood serum or plasma is suitable for a virus neutralisation test (Graham *et al.*, 2003).

Tissues for histological examinations should include gill, heart, pyloric caeca with attached pancreatic tissue, liver, kidney, spleen and skeletal muscle containing both red (aerobic) and white (anaerobic) muscle. Skin with associated skeletal muscle should be sampled at the lateral line level and deep enough to include both red and white muscle.

3.3. Samples or tissues not suitable for pathogen detection

Pancreas, although a target organ for the virus, is not suitable for RT-PCR detection of SAV, as it is impossible to separate this organ from the intestine of the fish during sampling, and in addition loss of pancreas is common in infected fish. Organs other than those recommended in Section 3.2. should not be used for the detection of SAV, as the sensitivity of the diagnostic methods might be reduced.

3.4. Non-lethal sampling

There are investigations into using non-lethal sampling methods for surveillance of SAV in fish farms, including detection of virus in water. However, no validated methods are currently available.

3.5. Preservation of samples for submission

For guidance on sample preservation methods for the intended test methods, see Chapter 2.3.0.

3.5.1. Samples for pathogen isolation

The success of pathogen isolation and results of bioassay depend heavily on the quality of samples (time since collection and time in storage). Fresh specimens should be kept on ice and preferably sent to the laboratory within 24 hours of collection. Alternate storage methods should be used only after consultation with the receiving laboratory.

Annex 16 (contd)

Before transfer to the laboratory, pieces of the organs to be examined should be removed from the fish with sterile dissection tools and transferred to sterile plastic tubes containing at least 4 ml transport medium, i.e. cell culture medium with 10% fetal calf serum (FCS) and antibiotics. The combination of 200 International Units (IU) penicillin, 200 µg streptomycin, and 200 µg kanamycin per ml are recommended, although other antibiotics of proven efficiency may also be used. The tissue in each sample should be larger than the analytical unit size required for initial laboratory testing (e.g. between 0.5 and 2 g) and taken in duplicate if retesting may be required.

Tubes containing fish tissues in transport medium for cell cultivation should be placed in insulated containers, such as thick-walled polystyrene boxes, together with sufficient ice or an alternative cooling medium with the similar cooling effect to ensure chilling of the samples during transportation to the laboratory. However, freezing of the samples should be avoided. The temperature of a sample during transit must never exceed 10°C.

Whole fish may be sent to the laboratory if the temperature requirements referred to in the first paragraph during transportation can be fulfilled. Whole fish should be wrapped up in paper with absorptive capacity and enclosed in a plastic bag. Live fish may also be transported to the laboratory.

The virological examination for isolation in cell culture should be started as soon as possible and no later than 48 hours after the collection of the samples. In exceptional cases, the virological examination may be started at the latest within 72 hours after the collection of the material, provided that the material to be examined is protected by a transport medium and that the temperature requirements during transportation can be fulfilled.

3.5.2. Preservation of samples for molecular detection

Samples can be taken from the fish in accordance with the procedure described in Section 3.5.1, using a sterile instrument, and transferred to a sterile plastic tube containing transport medium.

Alternatively, tissue samples for RT-PCR testing should be preserved in an appropriate medium for preservation of RNA. Samples in RNA stabilising reagents can be shipped on ice or at room temperature if transport time does not exceed 24 hours.

For further storage the samples can be kept at –20°C.

3.5.3. Fixed samples for histopathology, immunohistochemistry or *in-situ* hybridisation

Tissue samples for histopathology should be fixed in 10% neutral buffered formalin immediately after collection. The recommended ratio of fixative to tissue is 10:1.

3.5.4. Fixed samples for electron microscopy

Samples for electron microscopy are not routinely required and are collected only when it is considered beneficial to facilitate further diagnostic investigation. A 2 mm cubed section from each of the appropriate organs described in section 3.2 should be fixed in glutaraldehyde; the recommended ratio of fixative to tissue is 10:1.

3.5.5. Samples for other tests

Blood samples should be centrifuged for the collection of serum or plasma as soon as possible after sampling, to avoid lysis of the red blood cells. Serum or plasma samples should be shipped on ice to the laboratory to ensure virus viability.

3.6. Pooling of samples

The reliability of a virus isolation and real-time RT-PCR for detecting SAV in pooled samples from apparently healthy and clinically diseased populations of Atlantic salmon has been evaluated (Hall *et al.*, 2014). The results suggest that the use of individual samples rather than pools is more appropriate when testing for freedom from, or for confirmatory diagnosis of, infection with SAV (Hall *et al.*, 2014).

4. Diagnostic methods

The methods currently available for identifying infection that can be used in i) surveillance of apparently healthy populations), ii) presumptive and iii) confirmatory diagnostic purposes are listed in Table 4.1. by life stage. The designations used in Table 4.1 indicate:

Key:

- +++ = Recommended method(s) validated for the purpose shown and usually to stage 3 of the OIE Validation Pathway;
- ++ = Suitable method(s) but may need further validation;
- + = May be used in some situations, but cost, reliability, lack of validation or other factors severely limits its application;

Shaded boxes = Not appropriate for this purpose.

The selection of a test for a given purpose depends on the analytical and diagnostic sensitivities and specificities repeatability and reproducibility. OIE Reference Laboratories welcome feedback on diagnostic performance for assays, in particular PCR methods, for factors affecting assay analytical sensitivity or analytical specificity, such as tissue components inhibiting amplification, presence of nonspecific or uncertain bands, etc., and any assays that are in the +++ category.

UNOFFICIAL VERSION

Table 4.1. OIE recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method	A. Surveillance of apparently healthy animals				B. Presumptive diagnosis of clinically affected animals				C. Confirmatory diagnosis ¹ of a suspect result from surveillance or presumptive diagnosis			
	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV	Early life stages ²	Juveniles ²	Adults	LV
Wet mounts												
Histopathology ³					++	++	++					
Cytopathology ³												
Cell or artificial media culture					+	+	+		+	+	+	
Real-time RT-PCR	+++	+++	+++		+++	+++	+++		+++	+++	+++	
Conventional RT-PCR					++	++	++		++	++	++	
Amplicon sequencing ⁴									+++	+++	+++	
<i>In-situ</i> hybridisation												
Bioassay												
LAMP												
Ab ELISA												
Ag ELISA												
Immunohistochemistry (needs to be filled in by expert)												
Serum neutralisation assay		+	++		++	++	++					

LV = level of validation, refers to the stage of validation in the OIE Pathway (chapter 1.1.2); RT-PCR = reverse transcription-polymerase chain reaction methods; LAMP = loop-mediated isothermal amplification; Ab- or Ag-ELISA = antibody or antigen enzyme-linked immunosorbent assay, respectively

¹For confirmatory diagnoses, methods need to be carried out in combination (see Section 6). ²Early and juvenile life stages have been defined in Section 2.2.3.

³Histopathology and cytopathology can be validated if the results from different operators has been statistically compared. ⁴Sequencing of the PCR product.

⁵Specify the test used. Shading indicates the test is inappropriate or should not be used for this purpose.

4.1. Wet mounts

Not relevant.

4.2. Histopathology and cytopathology

The pathological changes most commonly found in clinically diseased fish are severe loss of exocrine pancreatic tissue, cardiomyocytic necrosis and inflammation, red (aerobic) skeletal muscle inflammation and white (anaerobic) skeletal muscle degeneration or inflammation. A less frequent but supporting finding is the detection of cells with many cytoplasmic eosinophilic granules along kidney sinusoids.

As the disease progresses, the development of these changes is not simultaneous in all organs: in a very short, early phase, the only lesions present might be necrosis of exocrine pancreatic tissue and a variable inflammatory reaction in the peripancreatic fat. Shortly thereafter, heart muscle cell degeneration and necrosis develop before the inflammation response in the heart becomes more pronounced. The pancreatic necrotic debris will seemingly disappear, and the typical picture of severe loss of exocrine pancreatic tissue will soon appear simultaneously with the increasing inflammation in the heart. Somewhat later, skeletal muscle degeneration, inflammation and fibrosis develop. In a proportion of fish, severe fibrosis of the peri-acinar tissue may occur, and in these cases, the pancreas does not recover (runts) (Christie *et al.*, 2007; Kerbart Boscher *et al.*, 2006; McLoughlin & Graham, 2007; Taksdal *et al.*, 2007).

Cytopathology is not relevant for diagnostic use.

4.3. Cell or artificial media culture for isolation

4.3.1. Cell lines

Isolation of field isolates of SAV in cell culture may be challenging (Christie *et al.*, 1998; Graham *et al.*, 2007b; Petterson *et al.*, 2013). CHSE-214 are commonly used for primary SAV isolation, but susceptible cell lines such as BF-2, FHM, SHK-1, EPC, CHH-1 or others, may be used. Variation in cell line susceptibility among different SAV field isolates has been reported (Graham *et al.*, 2008; Herath *et al.*, 2009), and it is therefore recommended that several cell lines are tested for initial cell culture isolation of SAV in a new laboratory or for a new virus strain. Cell lines should be monitored to ensure that susceptibility to targeted pathogens has not changed.

The CHSE-214 cells are grown at 20°C in Eagle's minimal essential medium (EMEM) with non-essential amino acids and 0.01 M HEPES (N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid) buffer, or Leibovitz's L-15 cell culture medium, both supplemented with fetal bovine serum (FBS) (5% or 10%) and L-glutamine (4 mM).

4.3.2. Sample preparation and inoculation

For virus isolation, cells are grown in tissue culture flasks or multi-well cell culture plates. SAV-positive controls may be inoculated in parallel with the tissue samples as a test for cell susceptibility to SAV. When positive controls are included, measures must be taken to avoid contamination.

i) Inoculation of cell monolayers

Prepare a 2% suspension of tissue homogenate or a 10% suspension of serum using L-15 medium or EMEM without serum, or other medium with documented suitability. Remove growth medium from actively growing monolayers (1- to 2-day-old cultures or cultures of 70–80% confluency) grown in tissue culture flasks or multi-well cell culture plates (see above). Inoculate monolayers with a low volume of the 2% tissue homogenate or 10% serum dilution (for 25 cm² flasks: 1.5 ml). Adjust volume to the respective surface area in use. Allow 2–3 hours of incubation at 15°C, followed by removal of the inoculum, and addition of fresh L-15 or EMEM medium supplemented with 2–5% fetal bovine serum (for 25 cm² flasks: 5 ml).

When fish samples come from production sites where IPNV is regarded as endemic, the tissue homogenate supernatant should be incubated (for a minimum of 1 hour at 15°C) with a pool of antisera to the indigenous serotypes of IPNV prior to inoculation.

Annex 16 (contd)

ii) Monitoring incubation

Inoculated cell cultures (kept at 15°C) are examined at regular intervals (at least every 7 days) for the occurrence of cytopathic effect (CPE). Typical CPE due to SAV appears as plaques of pyknotic, vacuolated cells. However, Norwegian SAV field isolates (both SAV3 and SAV2) usually do not produce CPE in low passages, and this is also reported for other SAV genotypes (Graham *et al.*, 2008; Petterson *et al.*, 2013). If no CPE has developed after 14 days, subculture to fresh cell cultures.

iii) Subcultivation procedure

14 days (or earlier when obvious CPE appears) after inoculation, the cultures are freeze–thawed at –80°C to release virus from the infected cells. The procedure can be repeated 1–2 times.

Following centrifugation at 3000 *g* for 5 minutes, the supernatants are inoculated into fresh cell cultures as described for the primary inoculation: remove growth medium, inoculate monolayers with a small volume of diluted supernatant (1/5 and higher dilutions) for 2–3 hours before addition of fresh medium.

Inoculated cell cultures are incubated for at least 14 days and examined at regular intervals, as described for the primary inoculation. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification, as described below. Cell cultures should always be examined for the presence of SAV by immunofluorescence (indirect fluorescent antibody test [IFAT]), as virus replication may occur without development of apparent CPE.

4.4. Nucleic acid amplification

4.4.1. Reverse-transcription, real-time polymerase chain reaction

The primers described below for real-time RT-PCR and RT-PCR with sequencing will detect all known genotypes of SAV.

RT-PCR may be used for detection of SAV from total RNA (or total nucleic acids) extracted from recommended organs or tissues (see Section 3.4). Real-time RT-PCR for the detection of SAV is recommended as it increases the specificity and also the sensitivity of the test.

For genotyping, RT-PCR with subsequent sequencing of fragments from the E2 gene is recommended.

The primers and probe sequences for real-time RT-PCR from the nsP1 gene, as well as primers for genotyping, are listed in Table 4.2. The E2-primers may also be used for conventional RT-PCR detection of SAV, if necessary. For RNA extraction, automatic and semi-automatic nucleic acid extractors can be used. In addition, a variety of manual RNA extraction kits can also be used successfully to extract SAV RNA. Various RT-PCR kits and qPCR machines can be used. The PCR programme depends on the kit and real-time PCR equipment used in the laboratory. The conditions for performing the real-time RT-PCR in the OIE Reference Laboratory is as follows: 50°C for 10 minutes, 95°C for 3 minutes, and 40 cycles of (95°C for 10 seconds, 60°C for 20 seconds). For the conventional RT-PCRs (sequencing), the following programme is used: 50°C for 30 minutes, 95°C for 15 minutes, and 45 cycles of (94°C for 60 seconds, 55°C for 45 seconds, 72°C for 60 seconds).

Table 4.2. Primers and probe sequences for RT-PCR and real time RT-PCR

Primer and probe sequences	Genomic segment	Product size	Reference
QnsP1F: 5'-CCG-GCC-CTG-AAC-CAG-TT-3' QnsP1R: 5'-GTA-GCC-AAG-TGG-GAG-AAA-GCT-3' QnsP1probe: 5'FAM-CTG-GCC-ACC-ACT-TCG-A-MGB3' (Tagman®probe)	QnsP1	107 nt	Hodneland <i>et al.</i> , 2006

Primer and probe sequences	Genomic segment	Product size	Reference
E2F: 5'-CCG-TTG-CGG-CCA-CAC-TGG-ATG-3' E2R: 5'-CCT-CAT-AGG-TGA-TCG-ACG-GCA-G-	E2	516 nt	Fringuelli <i>et al.</i> , 2008

UNOFFICIAL VERSION

Annex 16 (contd)

The following controls should be run with each assay: negative extraction control; positive template control; no template control.

4.4.2. Conventional PCR (PCR)

See Section 4.4.1. for comments on conventional PCR kits and PCR machines.

The following controls should be run with each assay: negative extraction control; positive template control; no template control.

4.4.3. Other nucleic acid amplification methods

Not applicable

4.5. Amplicon sequencing

Sequencing to determine the genotype of SAV can be performed using the E2-primer set listed in Table 4.2.

4.6. In-situ hybridisation

Not applicable

4.7. Immunohistochemistry

Immunohistochemical testing (Taksdal *et al.*, 2007) is only recommended for samples from fish with acute necrosis of exocrine pancreatic tissue.

4.7.1. Preparation of tissue sections

The tissues are fixed in neutral phosphate-buffered 10% formalin for at least 1 day, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard protocols. Approximately 3 µm thick sections (for immunohistochemistry sampled on poly-L-lysine-coated slides) are heated at 56–58°C (maximum 60°C) for 20 minutes, dewaxed in xylene, rehydrated through graded ethanol, and stained with haematoxylin and eosin for histopathology and immunohistochemistry as described below.

4.7.2. Staining procedure for immunohistochemistry

All incubations are carried out at room temperature and all washing steps are done with Tris-buffered saline (TBS).

- i) Nonspecific antibody binding sites are first blocked in 5% bovine serum albumin (BSA) in TBS for 20 minutes. The solution is then poured off without washing.
- ii) Sections are incubated with primary antibody (monoclonal mouse antibody 4H1 against E1 SAV glycoprotein [Todd *et al.*, 2001]), diluted 1/3000 in 2.5% BSA in TBS and then incubated overnight, followed by two wash out baths lasting a minimum of 5 minutes.
- iii) Sections are incubated with secondary antibody (biotinylated rabbit anti-mouse Ig) diluted 1/300 for 30 minutes, followed by wash out baths as in step ii above.
- iv) Sections are incubated with streptavidin with alkaline phosphatase 1/500 for 30 minutes followed by wash out baths as in step ii above.
- v) For detection of bound antibodies, sections are incubated with Fast Red⁶ (1 mg ml⁻¹) and Naphthol AS-MX phosphate (0.2 mg ml⁻¹) with 1 mM Levamisole in 0.1 M TBS (pH 8.2) and allowed to develop for 20 minutes followed by one wash in tap water before counterstaining with Mayer's haematoxylin and mounting in aqueous mounting medium.

Annex 16 (contd)

⁶ Reference to specific commercial products as examples does not imply their endorsement by the OIE. This applies to all commercial products referred to in this *Aquatic Manual*.

SAV-positive and SAV-negative tissue sections are included as controls in every setup (Taksdal *et al.*, 2007).

4.8. Bioassay

Not applicable.

4.9. Antibody or antigen-based detection methods

4.9.1. Antibody-based verification of SAV growth in cell culture

This technique should not be used as a screening method. All incubations below are carried out at room temperature unless otherwise stated.

- i) Prepare monolayers of cells in appropriate tissue culture plates (e.g. 96-well plates) or on cover-slips, depending on the type of microscope available (an inverted microscope equipped with UV light is necessary for monolayers grown on tissue culture plates). The necessary monolayers for negative and positive controls must be included.
- ii) Inoculate the monolayers with the virus suspensions to be identified in tenfold dilutions, two monolayers for each dilution. Add positive virus control in dilutions known to give a good staining reaction. Incubate inoculated cell cultures at 15°C for 9–11 days.
- iii) Fix in 80% acetone for 20 minutes after removing cell culture medium and rinsing once with 80% acetone. Remove the fixative and air dry for 1 hour. If necessary, the fixed cell cultures may be stored dry for 14 days at 4°C until staining.
- iv) Incubate the cell monolayers with anti-SAV MAb in an appropriate dilution in phosphate-buffered saline (PBS) for 1 hour and rinse three times with PBS with 0.05% Tween 20.
- v) Incubate with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin for 1 hour (or if the primary Ab is polyclonal from rabbits, use FITC-conjugated antibody against rabbit immunoglobulin), according to the instructions of the supplier. To increase the sensitivity of the test, FITC-conjugated anti-mouse Ig may be replaced with biotin-labelled anti-mouse Ig and FITC-labelled streptavidin with rinsing as in step d) in between the steps. The nuclei can be stained with propidium iodide (100 µg ml⁻¹ in sterile distilled water). Add PBS (without Tween 20) and examine under UV light. To avoid fading, the stained plates should be kept in the dark until examination. For long periods of storage (more than 2–3 weeks) a solution of 1,4-diazabicyclooctane (DABCO 2.5% in PBS, pH 8.2) or similar reagent may be added as an anti-fade solution.

4.10. Other methods

4.10.1. Immunoperoxidase-based serum neutralisation assay

Experimental studies have shown that neutralising antibodies can first be detected 10–16 days post-infection (Graham *et al.*, 2003), and serum neutralisation (SN) assays can be used as a diagnostic tool for the detection of SAV antibodies. SN assays are based on the presence or absence of detectable virus growth in cultured cells following incubation with serum that may contain neutralising antibodies. In addition, the assay allows detection of virus in serum or plasma, if present.

CHSE-214 cells are grown as described in Section 4.3.1 Cell lines. A suspension of trypsinised cells, diluted 1/3 in growth medium (10% FBS) is prepared for the SN assay.

- i) 1/20 and 1/40 dilutions of each test serum are prepared in maintenance medium (2% FBS), and transferred to two duplicate wells (15 µl per well) on a flat-bottomed tissue culture grade microtitre plate. An equal volume of virus (100 TCID₅₀ [median tissue culture infective dose]) is added and the plate is incubated for 2 hours at room temperature.
- ii) 70 µl of maintenance medium, and 50 µl of the CHSE-214 cell suspension is added to each well, and the plates are incubated for 3 days at 15°C.

- iii) The cell monolayer is then fixed and stained as described in Section 4.9.1 *Antibody-based verification of SAV growth in cell culture*, or using the following procedure: monolayers of CHSE-214 cells are fixed for 30 minutes at room temperature in 10% neutral buffered formalin. Following two washes with 0.01 M PBS, a MAb against SAV is added to the monolayers in an appropriate dilution. Bound MAb is visualised using a labelled streptavidin–biotin system according to the manufacturer's instructions.
- iv) SN titres (ND₅₀) are then calculated according to the method of Karber (1931), with titres $\geq 1:20$ being considered positive. Both serum controls (without virus added) and a virus control (without serum added) must always be included in the assay, to ensure valid results.

5. Test(s) recommended for surveillance to demonstrate freedom in apparently healthy populations

The recommended test to be used in surveillance of susceptible fish populations for declaration of freedom from SAV is real-time RT-PCR as described in Section 4.4.1. in this chapter.

6. Corroborative diagnostic criteria

This section only addresses the diagnostic test results for detection of infection in the absence (Section 6.1.) or in the presence of clinical signs (Section 6.2.) but does not evaluate whether the infectious agent is the cause of the clinical event.

The case definitions for a suspect and confirmed case have been developed to support decision making related to trade and confirmation of disease status at the country, zone or compartment level. Case definitions for disease confirmation in endemically affected areas may be less stringent. It is recommended that all samples that yield suspect positive test results in an otherwise pathogen-free country or zone or compartment should be referred immediately to the OIE Reference Laboratory for confirmation, whether or not clinical signs are associated with the case. If a laboratory does not have the capacity to undertake the necessary diagnostic tests it should seek advice from the appropriate OIE Reference Laboratory.

6.1. Apparently healthy animals or animals of unknown health status⁷

Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of animals or animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate disease freedom.

6.1.1. Definition of suspect case in apparently healthy animals

The presence of infection with SAV shall be suspected if at least one of the following criteria is met:

- i) Positive result by real-time RT-PCR
- ii) Positive result by conventional RT-PCR
- iii) SAV-typical CPE in cell culture
- iv) Detection of neutralising activity against SAV in serum or plasma.

6.1.2. Definition of confirmed case in apparently healthy animals

The presence of infection with SAV is considered to be confirmed if, in addition to the criteria in Section 6.1.1., one or more of the following criteria is met:

⁷ For example transboundary commodities.

- i) A positive result by real-time RT-PCR and a positive result by conventional RT-PCR and sequencing of the amplicon
- ii) SAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.2 Clinically affected animals

Clinical signs are not pathognomonic for a single disease; however, they may narrow the range of possible diagnoses.

6.2.1. Definition of suspect case in clinically affected animals

The presence of infection with SAV shall be suspected if at least one of the following criteria is met:

- i) Gross pathology or clinical signs associated with infection with SAV
- ii) Histopathology consistent with SAV infection
- iii) Positive result by real-time RT-PCR
- iv) Positive result by conventional RT-PCR
- v) SAV-typical CPE in cell culture
- vi) Detection of neutralising activity against SAV in serum or plasma.

6.2.2. Definition of confirmed case in clinically affected animals

The presence of infection with SAV is considered to be confirmed if, in addition to the criteria Section 6.2.1., one of the following criteria is met.:

- i) A positive result by real-time RT-PCR and a positive result by conventional RT-PCR and sequencing of the amplicon
- ii) SAV-typical CPE in cell culture followed by virus identification by conventional RT-PCR and sequencing of the amplicon.

Reference Laboratories should be contacted for specimen referral when testing laboratories cannot undertake any of the recommended test methods and testing is being undertaken that will result in notification to the OIE.

6.3. Diagnostic sensitivity and specificity for diagnostic tests

The diagnostic performance of tests recommended for surveillance or diagnosis of infection with SAV are provided in Table 6.3. This information can be used for the design of surveys for infection with SAV, however, it should be noted that diagnostic performance is specific to the circumstances of each diagnostic accuracy study (including the test purpose, source population, tissue sample types and host species) and diagnostic performance may vary under different conditions. Data is only presented where tests are validated to at least level two of the validation pathway described in Chapter 1.1.2. and the information is available within published diagnostic accuracy studies.

Table 6.1. Diagnostic performance of tests recommended for surveillance or diagnosis

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (n)	DSp (n)	Reference test	Citation
Real-time PCR	Diagnosis	Clinically diseased fish	Heart and mid-kidney	Atlantic salmon				Jansen et al, 2019
Isolation of SAV in cell culture	Diagnosis	Clinically diseased fish	Heart and mid-kidney	Atlantic salmon				Jansen et al, 2019
Detection of neutralising activity against SAV	Diagnosis	Clinically diseased fish	Serum or plasma	Atlantic salmon				Jansen et al, 2019
Histopathology	Diagnosis	Clinically diseased fish	Heart and mid-kidney	Atlantic salmon				Jansen et al, 2019

DSe = diagnostic sensitivity, DSp = diagnostic specificity, **n = number of samples used in the study** (this is pending further consultation with the expert), PCR: = polymerase chain reaction.

7. References

- ALDRIN M., STORVIK B., FRIGESSI A., VILJUGREIN H. & JANSEN P.A. (2010). A stochastic model for the assessment of the transmission pathways of heart and skeleton muscle inflammation, pancreas disease and infectious salmon anaemia in marine fish farms in Norway. *Prev. Vet. Med.*, **93**, 51–61.
- BANG JENSEN B., KRISTOFFERSEN A.B., MYR C. & BRUN E. (2012). Cohort study of effect of vaccination on pancreas disease in Norwegian salmon aquaculture. *Dis. Aquat. Org.*, **102**, 23–31.
- BRATLAND A. & NYLUND A. (2009). Studies on the possibility of vertical transmission of Norwegian salmonid Alphavirus in production of Atlantic salmon in Norway. *J. Aquat. Anim. Health*, **21**, 73–78.
- BRUNO D.W., NOGUERA P.A., BLACK J., MURRAY W., MACQUEEN D.J. & MATEJUSOVA I. (2014) Identification of a wild reservoir of salmonid alphavirus in common dab *Limanda limanda*, with emphasis on virus culture and sequencing. *Aquacult Environ Interact* 5:89-98. <https://doi.org/10.3354/aei00097>.
- CHRISTIE K.E., FYRAND K., HOLTET L. & ROWLEY H.M. (1998) Isolation of pancreas disease virus from farmed Atlantic salmon, *Salmo salar* L., in Norway. *J. Fish Dis.*, **21**, 391–394.
- CHRISTIE K.E., GRAHAM D.A., MCLOUGHLIN M. F., VILLOING S., TODD D. & KNAPPSKOG D. (2007). Experimental infection of Atlantic salmon *Salmo salar* pre-smolts by i.p. injection of new Irish and Norwegian salmonid alphavirus (SAV) isolates: a comparative study. *Dis. Aquat. Org.*, **75**, 13–22.
- FRINGUELLI E., ROWLEY H.M., WILSON J.C., HUNTER R., RODGER H. & GRAHAM D.A. (2008). Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. *J. Fish Dis.*, **31**, 811–823.
- GONEN S., BARANSKI M., THORLAND I., NORRIS A., GROVE H., ARNESEN P., BAKKE H., LIEN S., BISHOP S.C. & HOUSTON R.D. (2015). Mapping and validation of a major QTL affecting resistance to pancreas disease (salmonid alphavirus) in Atlantic salmon (*Salmo salar*). *Heredity*, **115**, 405–414. <https://doi.org/10.1038/hdy.2015.37>
- GRAHAM D.A., CHERRY K., WILSON C.J. & ROWLEY H.M. (2007a). Susceptibility of salmonid alphavirus to a range of chemical disinfectants. *J. Fish Dis.*, **30**, 269–277.
- GRAHAM D.A., FROST P., MCLAUGHLIN K., ROWLEY H.M., GABESTAD I., GORDON A. & MCLAUGHLIN M.F. (2011). A comparative study of marine salmonid alphavirus subtypes 1–6 using an experimental cohabitation challenge model. *J. Fish Dis.*, **34**, 273–286.

Annex 16 (contd)

GRAHAM D.A., FRINGUELLI E., WILSON C., ROWLEY H.M., BROWN, A., RODGER H., MCLOUGHLIN M.F., MCMANUS C., CASEY E., MCCARTHY L.J. & RUANE N.M. (2010). Prospective longitudinal studies of salmonid alphavirus infections on two Atlantic salmon farms in Ireland; evidence for viral persistence. *J. Fish Dis.*, **33**, 123–135.

GRAHAM D.A., JEWHRUST V.A., ROWLEY H.M., MCLOUGHLIN M.F. & TODD D. (2003). A rapid immunoperoxidase-based neutralization assay for salmonid alphavirus used for a serological survey in Northern Ireland. *J. Fish Dis.*, **26**, 407–413.

GRAHAM D.A., ROWLEY H.M. & FROST P. (2014). Cross-neutralization studies with salmonid alphavirus subtype 1–6 strains: results with sera from experimental studies and natural infections. *J. Fish Dis.*, **37**, 683–691.

GRAHAM D.A., STAPLES V., WILSON C.J., JEWHRUST H., CHERRY K., GORDON A. & ROWLEY H.M. (2007b). Biophysical properties of salmonid alphaviruses: influences of temperature and pH on virus survival. *J. Fish Dis.*, **30**, 533–543.

GRAHAM D.A., WILSON C., JEWHRUST H. & ROWLEY H. (2008). Cultural characteristics of salmonid alphaviruses – influences of cell line and temperature. *J. Fish Dis.*, **31**, 859–868.

Graham *et al.*, 2009

HALL, L.M., MUNRO, L.A., WALLACE, I.S., MCINTOSH, R., MACNEISH, K., MURRAY, A.G., (2014). An approach to evaluating the reliability of diagnostic tests on pooled groups of infected individuals. *Preventive Veterinary Medicine* 116, 305–312. <https://doi.org/https://doi.org/10.1016/j.prevetmed.2014.01.021>

HERATH T., COSTA J., THOMPSON K., ADAMS A. & RICHARDS R. (2009). Alternative cell line for the isolation of salmonid alphavirus-1. *Icelandic Agricultural Sci.*, **22**, 19–27.

HODNELAND K. & ENDRESEN C. (2006). Sensitive and specific detection of salmonid alphavirus using real-time PCR (TaqMan). *J. Virol. Methods*, **131**, 184–192.

JANSEN M.D., BANG JENSEN B. & BRUN E. (2014). Clinical manifestations of pancreas disease (PD) outbreaks in Norwegian marine salmon farming – variations due to salmonid alphavirus (SAV) subtype. *J. Fish Dis.*, **24** March, doi: 10.1111/jfd.12238

JANSEN M.D., GUARRACINO M., CARSON M., MODAHL I., TAKSDAL T., SINDRE H., BRUN E. & TAVORNPANICH S. (2019). Field evaluation of diagnostic test sensitivity and specificity for *Salmonid alphavirus* (SAV) infection and pancreas disease (PD) in farmed Atlantic salmon (*Salmo salar* L.) in Norway using Bayesian latent class analysis. *Frontiers Vet. Sci.*, doi: 10.3389/fvets.2019.00419

JANSEN M.D., TAKSDAL T., WASMUTH M.A., GJERSET B., BRUN E., OLSEN A.B., BRECK O. & SANDBERG M. (2010a). Salmonid alphavirus (SAV) and pancreas disease (PD) in Atlantic salmon (*Salmo salar* L.) in freshwater and seawater sites in Norway from 2006 to 2008. *J. Fish Dis.*, **33**, 391–402.

JANSEN M.D., WASWUTH M.A., OLSEN A.B., GJERSET B., MODAHL I., BRECK O., HALDORSEN R.N., HJELMELAND R., TAKSDAL T. (2010b). Pancreas disease (PD) in sea-reared Atlantic salmon, *Salmon salar* L., in Norway; a prospective, longitudinal study of disease development and agreement between diagnostic test results. *J. Fish Dis.*, **33**, 723–736.

JEWHRUST V.A., TODD D., ROWLEY H.M., WALKER I.W., WESTON J.H. MCLOUGHLIN M.F. & GRAHAM D.A. (2004). Detection and antigenic characterization of salmonid alphavirus isolates from sera obtained from farmed Atlantic salmon, *salmo salar* L., and farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.*, **27**, 143–149.

KERBART BOSCHER S., MCLOUGHLIN M., LE VEN A., CABON J., BAUD M. & CASTRIC J. (2006). Experimental transmission of sleeping disease in one-year-old rainbow trout *Oncorhynchus mykiss* (Walbaum), induced by sleeping disease virus. *J. Fish Dis.*, **29**, 263–273.

KONGTORP R.T., STENE A., ANDREASSEN P.A., ASPEHAUG V., GRAHAM D.A., LYNGSTAD T.M., OLSEN A.B., OLSEN R.S., SANDBERG M., SANTI N., WALLACE C. & BRECK O. (2010). Lack of evidence for vertical transmission of SAV 3 using gametes of Atlantic salmon, *salmo salar* L., exposed by natural and experimental routes. *J. Fish Dis.*, **33**, 879–888.

Annex 16 (contd)

KRISTOFFERSEN A.B., VILJUGREIN H., KONGTORP R.T., BRUN E. & JANSEN P.A. (2009). Risk factors for pancreas disease (PD) outbreaks in farmed Atlantic salmon and rainbow trout in Norway during 2003–2007. *Prev. Vet. Med.*, **90**, 127–136.

LEWISCH, E., FRANK, T., SOLIMAN, H., SCHACHNER, O., FRIEDL, A., EL-MATBOULI, M., 2018. First confirmation of salmonid alphavirus infection in Arctic char *Salvelinus alpinus* and in Austria. *Diseases of Aquatic Organisms* **130**, 71–76.

McLOUGHLIN M.F. & GRAHAM D.A. (2007). Alphavirus infections in salmonids – a review. *J. Fish Dis.*, **30**, 511–531.

NORRIS A., FOYLE L., RATCLIFF J. (2008). Heritability of mortality in response to a natural pancreas disease (SPDV) challenge in Atlantic salmon, *Salmo salar* L., post-smolts on a West of Ireland sea site. *J. Fish Dis.*, **31**, 913–920.

PETTERSON E., SANDBERG M. & SANTI N. (2009). Salmonid alphavirus associated with *Lepeoptheirus salmonis* (Copepoda: Caligidae) from Atlantic salmon, *Salmo salar* L. *J. Fish Dis.*, **30**, 511–531.

PETTERSON E., STORMOEN, M., EVENSEN O., MIKALSEN A.B. & HAUGLAND O. (2013). Natural infection of Atlantic salmon (*Salmo salar*) with salmonid alphavirus 3 generates numerous viral deletion mutants. *J. Gen. Virol.*, **94**, 1945–1954.

RODGER H. & MITCHELL S. (2007). Epidemiological observations of pancreas disease of farmed Atlantic salmon, *Salmo salar* L., in Ireland. *J. Fish Dis.*, **32**, 477–479.

RUANE N., GRAHAM D. & RODGER H. (2008). Pancreas disease in farmed salmon – health management and investigations at Irish farm sites 2005–2008. Marine Environments and Health Series, No. 34, Marine Institute. Available at <http://oar.marine.ie/handle/10793/267>

SNOW M., BLACK I., MCINTOSH R., BARETTO E., WALLACE I.S. & BRUNO D.W. (2010). Detection of salmonid alphavirus RNA in wild marine fish: implications for the origin of salmon pancreas disease in aquaculture. *Dis. Aquat. Org.*, **91**, 177–188.

STENE A., VILJUGREIN H., YNDESTAD H., TAVORNANICH S. & SKJERVE E. (2013). Transmission dynamics of pancreas disease (PD) in a Norwegian fjord: aspects of water transport, contact networks and infection pressure among salmon farms. *J. Fish Dis.*, **37**, 123–134.

STENE A., BANG JENSEN B., KNUTSEN Ø., OLSEN A. & VILJUGREIN H. (2014). Seasonal increase in sea temperature triggers pancreas disease in Norwegian salmon farms. *J. Fish Dis.*, **37**, 739–751.

STORMOEN M., KRISTOFFERSEN A.B. & JANSEN P.A. (2013). Mortality related to pancreas disease in Norwegian farmed salmonid fish, *Salmo salar* L. and *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.*, **36**, 639–645.

TAKSDAL T., OLSEN A.B., BJERKAAS I., HJORTAAS M.J., DANNEVIG B.H., GRAHAM D.A. & McLOUGHLIN M.F. (2007). Pancreas disease in farmed Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Norway. *J. Fish Dis.*, **30**, 545–558.

TAKSDAL, T. & SINDRE, H. (2016). Chapter 23 - Togaviruses of Fish, in: Kibenge, F.S.B., Godoy, M.G.B.T.-A.V. (Eds.), Academic Press, San Diego, pp. 357–364. <https://doi.org/https://doi.org/10.1016/B978-0-12-801573-5.00023-1>

TAKSDAL, T., BANG JENSEN, B., BÖCKERMAN, I., McLOUGHLIN, M.F., HJORTAAS, M.J., RAMSTAD, A., SINDRE, H., (2015). Mortality and weight loss of Atlantic salmon, *Salmo salar* L., experimentally infected with salmonid alphavirus subtype 2 and subtype 3 isolates from Norway. *Journal of Fish Diseases* **38**, 1047–1061. <https://doi.org/10.1111/jfd.12312>

THE NORWEGIAN SCIENTIFIC COMMITTEE FOR FOOD SAFETY (VITENSKAPSKOMITEEN FOR MATTRYGGHET) (2010). Risikouurdering - stamfiskovervåking og vertikal smitteoverføring. **01**, 1-44. Available at: [HTTPS://VKM.NO/DOWNLOAD/18.A665C1015C865CC85BDFC47/1500464589864/VURDERING%20AV%20SANNSYNLIG%20FOR%20OG%20RISIKO%20VED%20VERTIKAL%20OVERF%20C3%B8RING%20AV%20SMITTE.PDF](https://vkm.no/download/18.a665c1015c865cc85bdfc47/1500464589864/VURDERING%20AV%20SANNSYNLIG%20FOR%20OG%20RISIKO%20VED%20VERTIKAL%20OVERF%20C3%B8RING%20AV%20SMITTE.PDF)

Annex 16 (contd)

TODD D., JEWHRST V.A., WELSH M.D., BORGHMANS B.J., WESTON J.H., ROWLEY H.M., MACKIE D.P. & MCLOUGHLIN M.F. (2001). Production and characterisation of monoclonal antibodies to salmon pancreas disease virus. *Dis. Aquat. Org.*, **46**, 101–108.

VILJUGREIN H., STAALSTRØM A., MOLVÆR J., URKE H.A. & JANSEN P.A. (2009). Integration of hydrodynamics into a statistical mode on the spread of pancreas disease (PD) in salmon farming. *Dis. Aquat. Org.*, **88**, 35–44.

*
* *

NB: There is an OIE Reference Laboratory for infection with salmonid alphavirus (see Table at the end of this *Aquatic Manual* or consult the OIE web site for the most up-to-date list: <http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>). Please contact the OIE Reference Laboratories for any further information on infection with salmonid alphavirus

NB: FIRST ADOPTED IN 2014. MOST RECENT UPDATES ADOPTED IN 2019

UNOFFICIAL VERSION

© World Organisation for Animal Health (OIE), 2020

This document has been prepared by specialists convened by the World Organisation for Animal Health (OIE). Pending adoption by the World Assembly of Delegates, the views expressed herein can only be construed as those of these specialists.

All OIE publications are protected by international copyright law. Extracts may be copied, reproduced, translated, adapted or published in journals, documents, books, electronic media and any other medium destined for the public, for information, educational or commercial purposes, provided prior written permission has been granted by the OIE.

The designations and denominations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the OIE concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers and boundaries.

The views expressed in signed articles are solely the responsibility of the authors. The mention of specific companies or products of manufacturers, whether or not these have been patented, does not imply that these have been endorsed or recommended by the OIE in preference to others of a similar nature that are not mentioned.